

Hushing up Concorde

It is indisputable that Concorde is, by today's standards, a noisy plane on take-off and landing; no public relations exercise nor appeal to patriotism can allay the displeasure (at its mildest) that Concorde causes in the region of London Airport. But we are supposed to be objective scientists, and therefore not to resort to emotional phrases and subjective opinions when there exist adequate instrumental and numerical yardsticks.

The science of noise is still in a relatively primitive state, but there is general agreement between engineers, airlines and governments that the EPNdB scale (Effective Perceived Noise Decibels) is, at least for the present, an acceptable measure. This scale makes allowance for the duration of the noise but, as it is an average, obviously does not report short term fluctuations. Present noise standards at London Airport would require Concorde, weighing about 400,000 pounds on take-off, to generate no more than 107 EPNdB. Modern subsonic planes of comparable weight such as the DC-10 and L-1011 meet these standards with at least 6 dB to spare. Concorde seems, on average, to fail to meet the standards by several decibels. Figures generally indicating this have been issued both by the Department of Trade and by the Greater London Council within the past two weeks. Values on individual occasions fluctuate substantially; sometimes the noise is as much as 10 dB above the standards, whereas on 20 to 30% of flights the standards are satisfied.

Obviously, that is worrying enough in itself, but an equally major cause for concern is that such figures could have been withheld until such a ridiculously late stage in Concorde's development. It is absurd that extensive official documentation on noise appears only when planes have been painted in their owners' colours and bookings are being made for the first commercial flights, and, moreover, when the question of noise has become central for deciding whether there shall be operations into New York. Surely scientists and engineers could have made such predictions years ago.

They did. In 1972 Mr Michael Heseltine, then Minister for Aerospace, told the House of Commons the projections for 1975 noise levels for Concorde and subsonic jets. His figure of 114 EPNdB looks in hindsight about right, and did not look too offensive when placed alongside Boeing 707-320C and 747-100 figures. But what Mr Heseltine did not do—as Andrew Wilson pointed out in his book *The Concorde Fiasco* (Penguin, London, 1973)—was put Concorde's noise properly in context with the quietened versions of these noisiest subsonics. New models and retrofitted older planes were, even in 1972, achieving 6 dB reductions on the figures quoted for their noisier brothers. Between 1972 and 1975

there has been, any neighbour to an airport will testify, quite impressive noise reductions on subsonic planes. Concorde now sticks out like a sore thumb.

What then possessed those in charge of Concorde to soldier on, in the face of these depressing figures? The answer must have been a combination of immense technological momentum and confidence that regulations at London Airport would be no obstacle (though heaven knows where the plane would land) or a belief that something technological would turn up.

The only technological fix that would have been certain to reduce noise would have been to replace the Olympus engines (which are turbojets) with by-pass engines which, under names like turbofan and fan-jets, power many planes—including the supersonic Tu-144. But Olympus engines have been associated with Concorde from the very beginning, and design had been so carefully optimised around the Olympus that the idea was unthinkable. Beyond such a change, there is a very little. Talk of inexperienced pilots and improved antinoise procedures is a red herring. And devices such as the Thrust Reverser Aft and the spade silencer have not lived up to the promise that test-bed trials held out.

Blessed with hindsight, we can now see that in the early 1970s there were no well established grounds at all for believing that Concorde could meet airport regulations. This state of affairs is now confirmed just at the time that New York is looking at the noise problems.

The story has some rather unpleasant implications for the relationship between government and science and technology. Those who worked in noise were aware, years ago, that the Concorde problem was essentially insoluble, and that the chances of finding a palliative in a strictly limited time were negligible. And yet this message never got through to, or was ignored by, those who might, given a few years, have found political or administrative solutions which would have alleviated the present situation. "We couldn't write to the papers about it", one engineer told us, "quite apart from the risk of professional suicide, we knew that an immense public relations effort would be mounted to demonstrate how limited the horizons were bound to be of one man in one laboratory."

Whistle blowing is a risky enterprise, and many whistle blowers have suspect motives. But if the present Concorde problems are the result of a studied decision to override scientific and technical advice and simply to put on a brave face, perhaps one of the few good things that could come from this whole affair would be a more adequate exposure of the terms on which governments, and industries that governments encourage, use scientific advice. □





Aage Bohr: following in father's footsteps.

THE physics prize this year was awarded to Aage Bohr, Ben Mottelson and James Rainwater for their fundamental work on collective motion in nuclei. Bohr is already a famous name in physics, and Aage has now received the same distinction as his father Niels, who won the Nobel Prize in 1922 for his application of quantum theory to the hydrogen atom. This is not the first time a father and son have both received the physics prize: the Braggs received it jointly in 1915 for their work on X-ray crystallography, and the Thomsons for their work on the electron, 'J.J.' for discovering it in 1906 and 'G.P.' for showing its wave nature in 1937.

Bohr and Mottelson have worked together for many years at the Niels Bohr Institute in Copenhagen, and have maintained its reputation as one of the world's leading centres for theoretical physics, while Rainwater works at Columbia University in the USA.

The work that earned them the Nobel Prize forms one of the most recent chapters in four decades of effort to understand the atomic nucleus. The early work of Rutherford and his collaborators established that there is a tiny nucleus consisting of neutrons and protons. The problem is to understand how they are bound together, and hence to explain the observed features of nuclear reactions and nuclear structure.

We can learn something about the forces between the nucleons (a term denoting either protons or neutrons) by studying the collisions of individual nucleons with each other. The forces are certainly very complicated, and it is far too difficult to calculate the properties of the nucleus directly from them. But we do know that their net effect is to hold the nucleus together so we can represent this by an overall attractive potential parametrised by a depth and a radius. Unfortunately, calculations of nuclear properties from this model showed that some of them did not agree with experiment.

This extreme single-particle model, as it was called, was superseded in 1936 by the compound nucleus model of Niels Bohr, which took full account of the way the nucleons strongly interact. According to this model, a nuclear reaction takes place firstly by the capture of the incoming particles by the nucleus, followed by a relatively long period when its energy is shared and re-shared among all the nucleons. Finally by a statistical fluctuation enough energy is concentrated on a nucleon or group of nucleons near the nuclear surface to enable it to escape.

This model worked very well and was able to account for many nuclear

reactions, until in the 1940s it was found that many properties of the nucleons show marked changes whenever the number of neutrons (or protons) was one of the so-called 'magic' numbers 2, 8, 20, 28, 50, 82, 126, . . . This pointed to some sort of shell structure, as with atomic electrons, and these numbers were shown to follow readily from a model with a term effectively depending on the orbital angular momentum and the spin of the nucleons added to the central potential already considered. This potential has energy levels, which when filled with nucleons, one in each possible state to satisfy the Pauli exclusion principle, give automatically the magic numbers. For their independent discoveries of this Maria Mayer and Johannes Jensen were awarded the Nobel prize in 1963.

It was puzzling that the compound nucleus model requires the nucleons to interact strongly, while the shell model requires that they interact so weakly that they can follow relatively undisturbed orbits in the nucleus. This paradox is understood when we realise that the Pauli exclusion principle forbids most of the collisions that would otherwise take place within the nucleus because they would lead to states that are already occupied. A particle entering the nucleus from outside, however, has much higher energy so that the final states are seldom occupied and the interaction takes place strongly. This absorption was included in the simple models by allowing the potential to become complex at higher energies.

In all this work it was assumed that the nucleus is spherical, but data began to accumulate showing that some nuclei are quite markedly deformed. This evidence came mainly from their large quadrupole moments. Closed shell nuclei, in which the neutron and proton numbers are both magic, such as ^{16}O , ^{40}Ca and ^{208}Pb , are spherical, as is to be expected from the high symmetry of the closed shells. For other nuclei, the deformation increases with the number of nucleons outside the closed shells. It is notable that the quadrupole moment is always positive immediately before a shell is filled and negative immediately after. The quadrupole moments calculated from the orbitals only of the nucleons outside the closed shells were inadequate to explain to date.

In 1950 Rainwater suggested that the extra nucleons can polarise the core so that it becomes spheroidal. Thus the shape of the nucleus results from its own stabilising forces, tending to make it spherical, and the forces from the extra-core nucleons, which tend to deform it.

Why they were worth it

Peter Hodgson assesses the achievement of the physics Nobel prizewinners Bohr, Mottelson and Rainwater; and overleaf, Michael Stoker writes about the winners of the prize for medicine, Dulbecco, Temin and Baltimore.

If the nucleus is considered to be a liquid drop, the surface and Coulomb energies are proportional to the square of the eccentricity, for a spheroidal deformation. The effect of the distortion on the individual shell model orbits can be found by calculating the eigenvalues for a particle in a spheroidal potential, and together they give an energy that decreases linearly with the eccentricity e . Thus the total change in energy due to the distortion is

$$\Delta E \approx c_1 e^2 - c_2 e$$

and the stable shape for minimum energy is given by $e = c_2/c_1$. The constants c_1 and c_2 are known quite well, and Rainwater showed that this gives quadrupole moments that are similar to those found experimentally, and accounts for their variation with shell structure.

This vital suggestion removed the main difficulties about nuclear quadrupole moments, and provided the essential basis for a detailed theory of nuclear deformations. This was developed by Bohr and Mottelson and collaborators during the following decade. In their earlier calculations they treated the nuclear core as a charged, deformed drop of nuclear liquid interacting with the few nucleons outside the core. The motion of the core is described by a few dynamical variables, while the extra-core nucleons are treated individually. In later work they considered all the nucleons, and allowed them to move in a non-spherical potential, which represents the long range correlations between the nucleons.

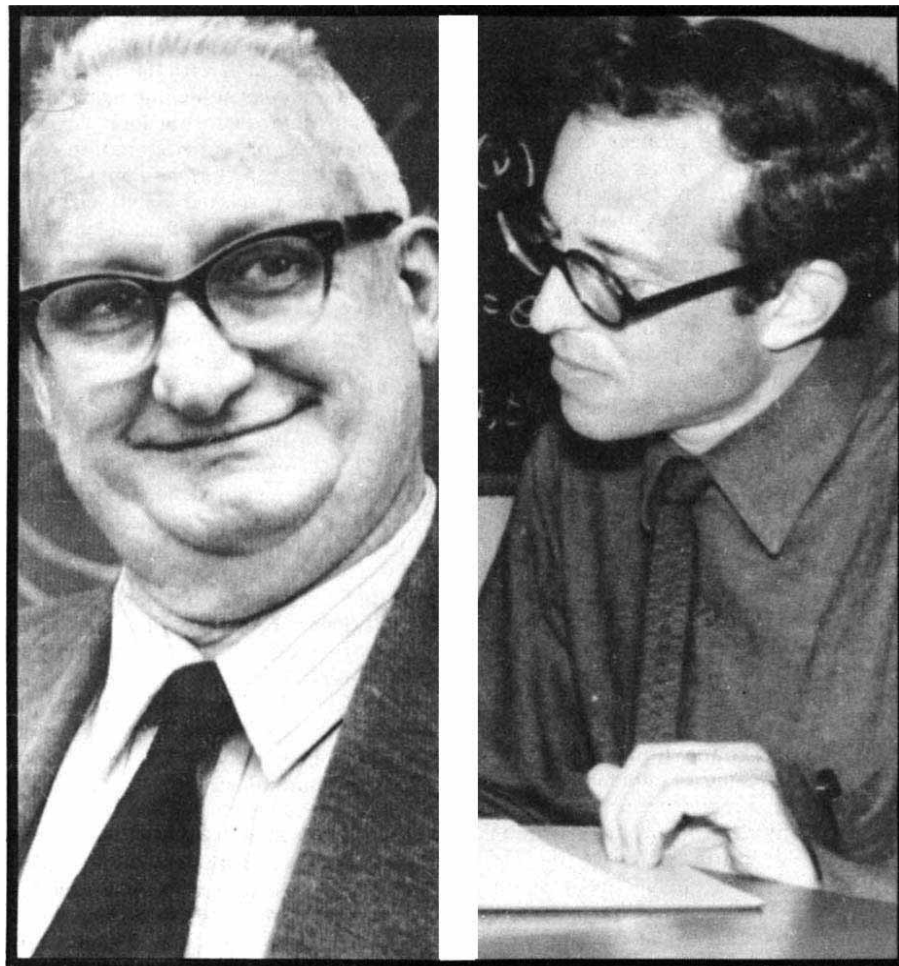
Bohr and Mottelson recognised that nuclear deformations can be of two types, static and dynamic, or more simply that nuclei are either hard or soft. Hard nuclei keep their shape, but if they are deformed they can be set into rotation, like a rotating rugby football. The energy levels of such a system can be calculated quantum mechanically, and are given by the formula

$$E = h^2 J(J+1)/2I$$

where I is the moment of inertia. In many nuclei, particularly the rare earths, whole series of rotational bands, each with many states, have now been identified. The theory, with its more detailed development, predicts these levels very accurately.

Soft nuclei, on the other hand, are easily given oscillations or vibrations, like the wobbling of a jelly. Calculated energy levels agree with those observed in some nuclei. The vibrational spectra are not as well marked as are the rotational spectra in hard nuclei because the vibrations are easily coupled to other types of motion, thus complicating the spectra.

One of the best known examples of nuclear collective motion is fission,



Rainwater (left) and Mottelson: prize for work on collective motion in nuclei.

when the nucleus breaks into two nearly equal parts. Niels Bohr did much of the early work on fission during the war years using the liquid drop model of the nucleus. In the 1950s Aage Bohr approached the problem from the microscopic point of view, in terms of the energy states of the fissioning nucleus. He showed that low energy fission takes place through very few reaction channels even though the level density is very high. This happens because most of the energy of the incoming neutron is spent on deforming the nucleus instead of exciting it internally. He has also made important contributions to the theory of the mass distribution of the fission fragments and of their angular distribution.

These rotational and vibrational models of the nucleus are known as collective models because unlike the independent particle or shell model for spherical nuclei they both require the nucleons to show collective or bulk motion. The nucleons are still moving rapidly along the independent shell model orbits but on a longer time scale the orbits change so that there is a resultant bulk motion of the nucleons, first in one direction and then in another, that forms the rotation or

vibration of the nucleus as a whole.

The great achievement of Bohr and Mottelson was to put all this work on nuclear collective motion on a sound and detailed quantum mechanical basis, and to apply the theories to account in detail for the properties of deformed nuclei throughout the Periodic Table. The hard nuclei are represented by a potential whose surface is expressed as a series of spherical harmonics, each with a coefficient giving a measure of the quadrupole, octupole, hexadecapole . . . deformations. The vibrations are similarly represented by dynamical deformation parameters that vary with time. All this work is described in detail in a monumental work on nuclear structure that Bohr and Mottelson have been writing for many years and which is being published in three volumes, the first of which appeared in 1969. These grew out of a series of lectures that is a continuing feature of the life of their institute. They are still full of activity, and will continue to contribute to our knowledge of nuclei, and to provide a source of stimulus and encouragement to younger workers in the years to come. □

Peter Hodgson

Nobel Prizes: 2

ALTHOUGH the pioneer discoveries on tumour viruses by Peyton Rous were begun in 1911, it is only in the past two decades that the particular importance of these viruses has been recognised, not only as causes of cancer, but as vital keys to the structure and function of animal cells. We now know that a prime feature of these tumour viruses is their ability to exist as integral parts of the chromosomes of their hosts, carried indefinitely from generation to generation in tumour cells, sometimes in normal cells, and even in germ cells, from parent to offspring. It is not surprising that the 1975 Nobel Prize for Medicine should be awarded to David Baltimore, Renato Dulbecco and Howard Temin, the three scientists whose discoveries revealed a phenomenon with such wide implications.

There can be few research workers with such a sustained harvest of discoveries as Renato Dulbecco. After graduating in medicine in Italy, and a period of initiation into phage genetics with Salvador Luria, he moved to Cal-Tech in 1952, and during the next few years, with Marguerite Vogt, his long standing collaborator, he set the scene for the whole new era of quantitative animal virology in cultured cells which has followed. This was begun by the development of the plaque assay, a precise and direct measurement of virus particles, making possible for the first time a detailed analysis of virus growth and virus antibody interaction, to say nothing of the isolation of genetically pure stocks of virus, an essential first step for the subsequent isolation of mutants. Much of this early work was with polio virus and provided the basis for the development of the live poliomyelitis vaccines.

In the late 1950s, however, interest in Dulbecco's laboratory was shifting to the tumour viruses, and it was here that Harry Rubin and Howard Temin (then a graduate student) first successfully applied Dulbecco's quantitative approach to the study of Rous sarcoma virus, by developing an assay of transformation, a cancer-like change induced by the virus in cultured cells. Meanwhile, Dulbecco and Vogt had concentrated on the recently discovered polyoma virus from mice, and in 1959 they reported transformation by this virus leading not only to abnormal growth of cells in culture, but also to the development of tumours when the cells were transplanted into animals. In the next few years, which included a sabbatical period in Glasgow, Dulbecco and his associates then went on

to characterise the DNA which constituted the genome of the virus. It was found to be a small circular molecule, and this alone was sufficient to produce the neoplastic transformation.

The studies of transformation and tumour induction by polyoma virus, and the closely related SV40 virus of monkeys, soon posed a crucial question concerning the fate of the virus, and its role, once the initial neoplastic event had taken place in an individual cell. Did the virus simply hit and run, or did it continue to multiply and persist in all the progeny cells? If the latter, was it essential for the continued abnormal behaviour of these cells? It was soon shown by Dulbecco and Vogt that the presence and growth of virus particles were not essential features of the transformed state, but there were indications, from the work of Habel and others on new cell surface antigens for example, that viral coded information might be present in the cells. The ring structure of the viral DNA had, in fact, suggested to Dulbecco that it might be incorporated into the chromosomal DNA of the host in a manner similar to prophage in lysogenic bacteria.

It was in 1967 and 1968, after moving to the new Salk Institute as a Founder Fellow, that Dulbecco and his associates produced the first evidence that this was, indeed, the case. First with Watkins the presence of a complete set of viral genes was demonstrated in apparently virus-free transformed cells, by fusion with a second 'permissive' cell which released the block in virus growth. Then with Westphal, and later with Sambrook and Srinivasan, molecular hybridisation was used to show that viral nucleic acid sequences were not only present in the transformed cells, but were chemically attached to the chromosomal DNA of the host. This fundamental series of discoveries, and others that followed, showed how, after the initial transformation or tumour induction, the virus might persist and so affect the behaviour of all the descendants of the original cell.

To investigate the role of the tumour virus genes in the cells, Dulbecco had begun the study of conditional mutants of the viruses, first of all at CalTech with his student, Fried, who isolated the *tsa* mutant of polyoma virus, and subsequently with Vogt and Eckhart at the Salk Institute, on a whole series of new mutants. Finally, as if this were not enough, Dulbecco has produced major contributions on the physiology of the transformed cells and their normal counterparts, work which has actively continued since he moved to London in 1972.

The discovery that a DNA-containing virus could persist by integration of its genome in an animal cell was

remarkable enough, but there was at least a model available in the integrated prophage of lysogenic bacteria. The RNA-containing tumour viruses were a different kettle of fish altogether—or so it seemed. Rous sarcoma cells, or cells transformed in culture by this and similar viruses, may admittedly harbour virus particles, but since the viruses contain RNA alone there seemed to be no way in which the genetic information could be transferred into DNA to allow a stable relationship with the chromosomes of the host cells. Nevertheless, the virus-cell relationship was clearly very stable, and there were preliminary indications that some of the RNA tumour viruses could be transmitted in chromosomes from parent to offspring in Mendelian fashion. It was this problem which attracted Howard Temin.

Temin had continued his early work with Rous sarcoma virus, and using virus mutants made the interesting finding that the virus could determine cell morphology. After he left Dulbecco's laboratory he moved to the McArdle Institute at Madison, Wisconsin, and there began to study the nature of the provirus, as he called it, in transformed cells. After showing that DNA synthesis was a necessary requirement for transformation, he went on to search for viral DNA in the transformed cells. Soon came his scandalous proposal that the genetic information in tumour virus RNA was first transcribed into DNA, which was, in turn, incorporated in the transformed cells. This was not, as some have suggested, an offence to Crick's central dogma, but it was, none the less, pretty unorthodox. Indeed, when Temin's first nucleic acid hybridisation data, purporting to show Rous virus DNA sequences in transformed cells, were published in 1964, they were not very convincing and there was a good deal of sad head shaking. In time, Baluda produced more convincing evidence for viral DNA sequences in transformed cells which seemed to support Temin's views, but the principal stumbling block was the absence of any known enzyme system which could make a DNA copy from an RNA molecule, and it was this problem which Temin began to investigate.

Meanwhile, in 1968 David Baltimore had come to work in Dulbecco's laboratory. While there, and later at MIT, he made a series of important discoveries on the replicating form of the RNA of poliovirus, and on the processing of the newly synthesised virus proteins. He had not worked with tumour viruses, but was presumably influenced by the work going on around him in Dulbecco's laboratory, and by Baluda's recent hybridisation data. After he left to take up his position in

Boston, he began, like Temin, to look for an enzyme in Rous sarcoma virus which would synthesise DNA on a RNA template.

And so it turned out that, simultaneously but quite independently in May 1970, Baltimore, and Temin with his colleague, Mizutani, reported the phenomenon which soon became known as reverse transcription. In elegantly simple experiments they both showed that radioactive precursors were incorporated into DNA in Rous sarcoma virus particles containing RNA alone, and this DNA turned out to be complementary to the virus RNA. Baltimore and his colleagues have since gone on to investigate in considerable detail the activity of reverse transcriptase, the enzyme concerned, and in a series of outstanding papers have described most of the steps by which double-stranded DNA is constructed from virus RNA.

Many of the implications of the discovery of reverse transcription were

immediately obvious and were breathtaking in their scope. It immediately vindicated Temin's hypothesis that RNA tumour viruses persist in tumour cells in the form of DNA copies, and it was not long before Hill and Hillova demonstrated infectious DNA in Rous virus-transformed cells. This linked the work of Baltimore and Temin to that of Dulbecco, and permitted a unified view of tumour viruses in general, since they are clearly all DNA viruses at heart, at least in their stage of association with the host cells. In addition, the discovery of reverse transcriptase has itself provided an extraordinarily powerful new tool, since it makes possible the construction of highly labelled complementary DNA molecules which can be used as probes to search for similar sequences in either DNA or RNA. This has led, not to new and sensitive methods in the search for human tumour viruses, particularly those developed by Spiegelman, Gallo, and others, but

also to more general studies on messenger RNA and its relationship to the chromosomal DNA, for example, in studies on the haemoglobin and γ globulin genes. Finally, the demonstration that the flow of genetic information from DNA to RNA can be reversed has provoked a whole range of new and exciting speculations (and even experiments) on the relationships of viruses and their hosts, not least with Temin's own provoking ideas about the 'normal' role of proviruses in gene amplification and differentiation.

The discoveries by this year's prize-winners have opened entirely new prospects in virology and cancer studies, and have added a quite new dimension to molecular biology. Had he survived, Peyton Rous, who started it all and who waited 55 years for his Nobel Prize, would have been the first to express his delight at the Committee's choice.

M. G. P. Stoker

international news



THE two Venus probes, Venera 9 and 10, which soft-landed successfully on October 22 and 25, respectively, at sites some 2,200 km apart, have resulted in considerable rethinking of many ideas about the planet. The members of the Soviet Venus project, faced with pictures of sharp, angular, granitelike rocks (see picture above) instead of the expected sand, have been quick to comment. The sharp, "young appearance of the rocks" said mineralogist Aleksandr Vasilevskii, indicates recent seismic or volcanic activity, typical of a "live" planet. The sharp shadows, indicative of sunlight filtering through the cloud cover to the surface and the flat landscape, rather than the expected "gold-fish bowl effect" produced by postulated atmospheric distortion, have considerably opened up the possibilities of further visual observation of the surface, since it is now clear that the

Venus, courtesy of Moscow

from Vera Rich

photographs obtained can be considered and interpreted as received, without the need for empirical correction and compensation. M. Ya. Marov, of the Long-Range Space Communications Centre, spoke of the photographs as inaugurating a new era in space research, in which "clearly directed complex investigations" would replace the somewhat hit-and-miss efforts of the previous "age of reconnaissance". In the same mood of euphoria, *Pravda* also speculated on the possibility of manned Venus missions in the next century.

Meanwhile, the remaining data

gathered and transmitted by the probes, including analysis of the cloud cover and the optical characteristics of the atmosphere, has gained relatively little attention, and must await routine processing.

● The Soviet Minister of Education, Mr V. P. Elyutin, visited Britain and, more particularly, the Open University last week. This visit, clearly an exercise in *detente*, yielded little definite outcome, although some fields of cooperation between the Open University and its Soviet opposite number are envisaged. Mr Elyutin spoke of the possible mutual publication of materials, and mutual production of television programmes, and also joint research work on common problems such as environmental protection and also special research into the theoretical problems of higher education.

Speaking of Soviet higher education,

WHEN a federal court ruled last year that grant proposals submitted to the National Institutes of Health (NIH) should be made public on demand, a number of scientists, alarmed at the possibility that their research ideas could be plagiarised, went scurrying to Congress seeking a bill to override the court's decision. They have not had a very sympathetic hearing on Capitol Hill, however. It now seems that the best they can hope for is that their concern will be investigated by an independent commission.

Several scientific and academic groups, led by the Association of American Medical Colleges (AAMC) have been lobbying hard for a provision, designed to preserve the confidentiality of grant applications for at least a year after they have been funded to be attached to a bill extending the programmes of the National Heart and Lung Institute. But last week, the House of Representatives passed the bill with only a mild provision directing the President's Biomedical Research Council—an influential commission which is now examining NIH policies—to look into the matter and report by next May. A Senate subcommittee, chaired by Senator Edward Kennedy, will approve its own version of the heart and lung research bill in the next week or so,

but it is not expected to deal with the confidentiality issue at all.

It therefore seems likely that all the details contained in grant proposals will continue to be made available to anybody who goes to the NIH and asks to see them. Though NIH lawyers have

Science no secret, yet

by Colin Norman, Washington

interpreted the court's decision narrowly by insisting that it applies only to proposals which have been funded or which are up for renewal, many scientists are concerned that it could open the door to some trouble.

First, there is the problem of possible plagiarism. Grant applications are supposed to describe exactly how the proposed experiments will be carried out. The court ruling would make such details publicly available as soon as the grant is awarded, rather than when the final results are reported, and scientists are therefore concerned that their competitors could obtain complete details of what they are doing, carry out the experiments themselves, and rush into print before the hapless originator of the idea had been able to do so.

The court dismissed such notions, however, by pointing out that it makes

no difference whether or not "biomedical scientists are really a mean-spirited lot who pursue self-interest as ruthlessly as the Barbary pirates did in their own chosen field", because the law—in this case the Freedom of Information Act—clearly applies.

The AAMC is also worried that the court ruling could upset the peer-review process by which grant proposals are evaluated. The association contends that grant applicants will be reluctant to describe their proposed experiments in detail, and funding decisions will therefore be made by peer reviewers on the basis of sketchy information.

But Congress has been reluctant to step in and exempt grant applications from public disclosure for a number of reasons. For one thing, the Freedom of Information Act is an admirable piece of legislation which has ensured that much public information is actually made public, and legislators are therefore anxious not to weaken it. And for another, there has recently been considerable discussion in the United States of the ethics of various experiments on human subjects, and Congress is determined to ensure that details of such experiments are made publicly available so that ethically questionable studies cannot be hidden behind a cloak of secrecy.

he reported that there is no decrease in the number of scientists being trained in the USSR, since the proportion of each discipline in each year's turnout of students is held constant and at present the total student population is increasing.

● The Jubilee Celebrations of the Soviet Academy of Sciences, which, after a postponement of more than a year, finally took place in the first half of October, following a fairly predictable pattern.

Of far more significance was the press conference given by Dr James C. Fletcher of NASA, while in Moscow for the celebrations. As part of the Soviet-US cooperation in Space research, Dr Fletcher revealed, talks were going on to explore the possibility of exchanging ground equipment enabling each partner to monitor the weather and research satellites of the other. In addition, in exchange for data from the American Landsat satellites, which monitor natural resources, the Soviets would provide the USA with similar data from high altitude reconnaissance aircraft, which use similar techniques of spectral analysis to gather geographical data. One interesting sidelight on the proposal is that such an exchange would throw a clearer light on to the Kosmos satellite programme. Since its inception in 1962,

this has been a useful cover-all for miscellaneous objects in space which could not be otherwise explained; not only the inevitable military reconnaissance satellites, but also failed interplanetary probes, unpublished trial runs of new spacecraft and so on. Although many objects have been identified over the years by outside observers, the Soviet space planners have never commented on the identifications, although data gathered by certain named Kosmos satellites are, from time to time, published in the technical literature. If this exchange of equipment goes forward, some new designation of satellites might become necessary. Weather forecasting is already the responsibility of the 'Meteor' series. Perhaps, rather than making certain Kosmos satellites available to the Americans, a new series of geophysical satellites might be inaugurated under another name.

The postponement of the Academy celebrations from May 1974 to October 1975 was itself something of a mystery. The official explanation was that the celebrations would coincide with the Soviet elections; furthermore, that more time was necessary for the preparations at all levels of Soviet scientific society. This second reason was echoed in the press conference given on September 25 by the new President of the

Academy, Vladimir A. Kotelnikov, who spoke of "all jubilee measures" being now complete. Nevertheless, at the time of the postponement, these excuses were felt to be incomplete. Possibly the illness of the then President of the Academy, Mstislav V. Keldysh, who resigned last May, played its part. A number of observers, however, felt that the postponement might not be unconnected with the whole problem of intellectual and academic freedom in the Soviet Union, and the possible embarrassments which might be caused should visitors from abroad refer to such questions. Whatever the truth of the matter, the three dissident members of the Academy, Andrei D. Sakharov, Igor R. Shafarevich, and Veniamin G. Levich, did receive invitations for the jubilee celebrations, thus forestalling any possible criticisms on their account.

This atmosphere of goodwill was, however, short-lived. Following the announcement that Sakharov was to receive the Nobel Peace Prize, the Tass agency began a campaign of attack directed against both Sakharov and the Peace Prize adjudicators. On October 13, Levich, who had been promised an exit visa for Israel by the end of 1975, was informed that such a visa would not, in fact, be forthcoming. □

A REGULAR visitor would have been surprised: all the parking places were taken. Latecomers had squeezed their cars into corners, rolled them on to the raked gravel paths and finally let them spill down the drive. Coming to the Royal Swedish Academy of Science for the first of last week's Nobel Prize announcements was rather exciting. The second and third times were less so. It only took one visit to realise that the parking jam was not caused by eager newsmen, but by sedate Academicians meeting in traditional session; that the low murmur heard in the imposing lobby was not because the crowd was tense and suppressed, but because it was very small; and that the single flex snaking up the stairs, pregnant with promises of a media extravaganza, led in fact to the only microphone present.

Could it, realistically, have been otherwise? Like all businesses, the media invests large amounts of resources only with the prospect of a payoff. And today's Nobel Prizes for Science do not make newspapers sell. Gone are the days when the names of even scientific laureates were likely to be household words. Gone even are the days when scientists active in the same subject were certain to know of the winners. What physicist working before the mid-1950s had never heard of Fermi, Blackett or Cockcroft? Today's laureates are unlikely to be known outside their own specialisations.

This is not to question the distinction or value of the contributions made by the present prizewinners. It is merely to recognise the greatly increased scope of scientific endeavour, the pervasiveness of specialisation and the replacement of the individual scientist working in his laboratory by the team. Small wonder that the old doyens, known by all in the subject, no longer exist.

It should not really have been surprising to find so few newsmen there. The trends which have influenced the development of science have, after all, also left their mark on the reporting of it. The scope and teamwork of newsgathering has been dramatically broadened by the wire services. What role can be played by the individual pressman—be he ever so enthusiastically crouched over his telephone—in the face of the news giants whose technology will have bounced the headlines across the globe almost before he has had time to dial his number? Those few reporters who went to the Academy's announcements swapped stories about how it used to be when news sleuths concocted elaborate schemes to get the laureates' names out first: when they held lines open in local telephone boxes and waited for

the names to be communicated by pre-arranged signals from collaborators inside the Academy building—and what happened once when a man sufficiently like the key informant to be mistaken for him came out and innocently lit a cigarette, thereby putting into action an intricately planned (and later highly embarrassing) series of events.

A prize system which rewards individuals when teamwork dominates scientific research, and which rewards so few individuals when so many are now in the field, is bound to cause some

Letter from Sweden

from Wendy Barnaby, Stockholm

disenchantment. In spite, and partly because, of this, the distinction of being a science laureate is as high as ever. If the reporters' indifference is easy to understand, so also is the winners' delight.

● Sweden's ambitious nuclear energy programme is amply underpinned by the country's uranium resources, which are estimated to be the West's largest in the price range (1968 figures) of \$10–15 a pound (cheaper uranium can be mined for less than \$10 a pound). Knowing it is there is not the same as getting it out, however. The State Power Board, the government-owned nuclear energy research company AB Atomenergi and the mining firm LKAB are keen to start extracting uranium from 15 km² around South Billingen, about 350 km south-west of Stockholm. But they are not the only ones with their eyes on the area. To begin with, the region is populated and the soil is good for agriculture and forestry. Cultural historians prize it for its dozens of ancient monuments (including five gravesites dating from the Iron Age). In view of this, the military's interest seems particularly callous: they want to use it for a shooting range. And there has also been talk of the suitability of the land for limestone mining.

● Sweden and Yugoslavia are expanding their scientific cooperation. Under an agreement signed recently in Stockholm, scientists and experts from the two countries will exchange visits of 10 person-months a year, symposia will be held and the traffic of scientific and technical journals enlivened. A spokesman for the Royal Academy of Engineering Sciences, which negotiated the agreement, said that the specific areas of cooperation had not yet been decided but that they would be in applied rather than basic science.

The agreement is similar to those Sweden has with other East European countries in that, although each sig-

natory can nominate scientists it would like to host, the final decision rests with the other's authorities. In this sense it is a less exciting arrangement than that concluded this year between the Swedes and the Russians, in which each undertook to send the specific experts requested by the other. In practice, of course, this aspect of the undertaking may make very little difference to the work actually done. For the moment, it is enough that each side wants the volume of this work to increase.

● Asbestos, and its use in working environments, is the subject of new regulations issued by the Swedish Work Protection Board. In the face of increased consciousness of the dangers of exposure to asbestos dust, the victims of which at present number about 200 (including some cases of lung cancer), the new regulations aim to minimise its use, to regulate unavoidable use and to educate the users to the dangers involved.

Sweden uses about 30,000 tons of asbestos annually, half of it raw and half in the form of ready-made products such as asbestos cement. Although its use is being discontinued where substitutes can be found—as in the manufacture of torpedo boats—it seems difficult to replace it totally with safe materials. Brake linings, for example, still depend on asbestos to withstand great heat. Given this situation, total protection of the working population from this particular environmental hazard still looks to be some way off.

● Taking measures to improve working conditions is not Sweden's only activity in this area. "The working environment" is one of the obligatory subjects for students beginning university-level education in trade techniques. This autumn, eight trial courses in the techniques of the clothing, food, paper, forestry, workshop and steel industries are being held around Sweden for people who have had basic training and at least four years of employment in one of these fields. The idea is to educate experienced workmen for more responsible positions—vocational trainers, production and control technicians, foremen—within the various industries.

At Eskilstuna, to the west of Stockholm, 30 employees from different workshops—among them, sheet-metal workers and tool-makers—are starting a course that will demand 40 hours a week for three terms. Besides "the working environment", they are taking off with six subjects: Swedish, physics, mathematics, materials, drawing and organisation. There seems to be no uncertainty about their employment prospects after the course. □

THE Arab League Educational, Cultural and Scientific Organisation (ALECSO) and the United Nations Environment Programme (UNEP) have started a Regreening of Arab Deserts Programme. Task forces of consultants have visited Arab countries to interview leaders of scientific projects, heads of government departments concerned with land reclamation plans, and institutes of research concerned with desert studies and with aspects of land use in arid territories.

Their aims are to identify national experiments for applying science and technology to the fight against desert creep and to the reclamation of desert lands, the conservation of surface deposits, the use of saline water for irrigation, the improvement of technologies for 'subsurfacing' with special materials including asphalt and asphalt-like substances, the conservation of natural pasture and experimental range management, animal breeding for improved stock, sand dune stabilisation, farming in desert oases (underground water resources) and to the integrated development of natural resources and nomads.

A coordinated intergovernmental research programme, in which the Arab countries will participate, will use the information and data provided by the task forces as a basis for consultations on the scientific content, plan of action and budget of the programme. The aim is the rational use of scientific knowledge and technical innovations in halting desert creep which is now proceeding at an alarming rate, and regreening extensive tracts of man-made deserts. Also planned are a study of human impact on nature and natural resources in Arab countries and a survey of the existing national institutional arrangements dealing with deserts.

Task forces have already prepared interim reports on their visits. In Libya they found that trees planted in open sand have little chance of survival because the movement of the sand by the wind in summer, when the soil is dry, either buries them, uncovers the roots or sandblasts them. So temporary stabilisation is carried out by spraying with a petroleum mulch heated to a temperature of 40–60 °C. The cost of spraying 1 hectare is about 120 Libyan pounds (\$375).

In Saudi Arabia, a number of nurseries have been established on a total area of about 18 acres, producing 600,000 saplings a year (comprising the native *Tamarix gallica* and *T. aphylla*, and such introduced species as *Eucalyptus camaldulensis*). The result was that sand dune encroachment from the north and north-east was checked and 14 villages menaced by sand dunes have been protected. Afforestation amounts

to 1,250 acres (10 million trees) and other projects include the drainage of swamps and water-logged ground, and the establishment of a national park.

A land reclamation project has been started at Taourgha, 200 km east of Tripoli and about 25 km south of the Libyan coast. Irrigation is by artesian water from the Taourgha spring, which flows freely at the rate of 3.0–3.25 m³ s⁻¹ or about 250,000 m³ d⁻¹ and contains about 2,500–3,000 p.p.m. of soluble salts (that is, class 4 water, with very high salinity). The total area involved in the project is 3,000 hectares,

The Arab World

from Salah Galal, Cairo

and the growth of crops (alfalfa and barley) has been quite satisfactory with water containing 3,000 p.p.m. of dissolved salts. The soil is quite permeable, the drainage is adequate, and irrigation water is applied in excess to provide considerable leaching (50% leaching requirement).

In Tunisia the government established in 1962, with assistance from the UN Development Programme and UNESCO, a Research Centre for the Utilisation of Saline Water Irrigation (CRUESI) to study the prospects of irrigation with saline water and to train specialists and technicians. CRUESI has six field stations representing a range of soil conditions, water quality and climate, and has in hand a series of well designed experiments combining various water quantities, soil treatments, land preparation and crops. Tested crops included alfalfa, clover, maize, beans, pimientos, rye grass, watermelons, sugarbeet, artichokes, fodder sorghum, tomatoes and cotton. The experiments carried out since 1962 have already produced valuable scientific information.

The ALECSO/UNEP task force of consultants has recommended a co-ordinated research programme to restore and manage rangelands in Arab countries, with studies on the recovery and succession of vegetation types and changes in biomass and production with regard to grazing; selection of useful native species from the point of view of their occurrence, phenomenological development and ecological relationship; the improvement of practices like range pitting and contour furrowing; studies to control desert creeping and sand dune shifting (by such methods as mulching, wind breakers and the placing of brush barriers); wind strip cropping control; and the limitation of soil erosion by wind and water.

● Professor Mahmoud Mohamed, professor of radiotherapy and nuclear

medicine at Cairo University and ex-Minister of Public Health in Egypt, has put forward a strategy for cancer control in developing countries, with Egypt as a model. It stresses the inadequacy of basic and central health information and documentation centres (which means that assessing health problems is usually a matter of empirical estimation), the inadequacy of resources and investments for upgrading existing services and research so that the attention of the health administration is usually focused on curative rather than on preventive services; and the lag which usually occurs between health development schemes and manpower development. Suprastructure development in cancer research is usually not planned or linked to existing health priorities, says Professor Mohamed, and furthermore, it suffers from factors as diverse as a brain drain, low socio-economic standards, and illiteracy—a pertinent feature of underdevelopment which impedes the extension of health education. Short life expectancy and high infant mortality rates add to the problems of cancer control in Egypt, with the real incidence and mortality rate still unknown and information available only in the form of relative frequency data. The estimated number of new cases each year is about 10,000–12,000 (Cairo University Hospital 200; Cancer Institute 2,000; Alexandria University Hospitals 1,500; Ain Shams University Hospitals 300; Mansoura University Hospitals 200; Tanta University Hospital 530; Assiut University Hospitals 120; Ministry of Public Health Hospitals 500; private cases 3,800.)

The most common cancer, in the male, is that of the urinary bladder (21–24%) and the most common in the female is cancer of the breast (20% of all female cancer cases attending the radiotherapy department of the Cairo University Hospitals). Most patients present themselves in the late stages of their disease to the 10 treating centres (all of which are teaching centres) or to 40 general hospitals and 159 district hospitals with inadequate or old equipment.

The proposed strategy includes decentralisation of the diagnosis of cancer; centralisation of treatment, manpower development and research in university centres; relying on the GP and his team for early detection; concentrating research resources on epidemiological aspects as a priority; establishing the registration of cases as an integral part of a general health registry; and the establishment of cancer control teams in teaching hospitals.

● UNESCO plans to convene a Conference of Ministers in Arab States Responsible for the Application of

Competition 2. Limericks to illustrate scientific principles. An enormous crop; we haven't verified that the selection that we print are original, and in at least one case the science is wrong (no prizes for pointing out errors). £10 to G. J. S. Ross of Harpenden, Hertfordshire; a clear winner with black holes, and also runner up with plate tectonics.

A pedantic astronomer said—gravity
Is causing semantic depravity.

These stars may be black

As light outflow they lack,

But holes? there's no room for a cavity.

G. EDWARDS

There once were two frogs of Gondwana
Who vowed ne'er to part till Nirvana,

But each met its fate

On a separate plate:

He lies in Brazil, she in Ghana.

G. J. S. ROSS

"Apollo to Mission Control—

We are almost in reach of our goal,

But our readings of G

Seem excessive to me,

So we may be inside a black ho . . .

G. J. S. ROSS

Poor John could not savor a rose

Despite hard proboscis blows

"My genes", explained he,

"Have an A for a G

So I suffer a code in the nose."

A. MEHLER



A plumber by name of Fred Slaughter,
With a wife and extravagant daughter,
Shouted, "Praise and acclaim
For whatever's to blame

For the anomalous expansion of water."

I. P. FREEMAN

A Greek once, in Physics a seeker,
Exultantly shouted "Eureka!"

He leapt from his bath

And rushed down the path—

Archimedes, the prototype streaker.

J. N. F. JURITZ

Though *coli* may bother and vex us,
It's hard to believe they outsex us.
They accomplish seduction
By viral transduction,
Foregoing the joys of amplexus.

S. GILBERT

While searching Mt. Aetna for data,
Old Pliny once tripped on a crater.
His feet couldn't hold.

Down the mountain he rolled

At a speed nearly (g) (sine of theta).

S. GILBERT

Competition No 3

Archimedes is said to have discovered his most cherished principle in the course of messing up the bathroom floor, Newton upon being disturbed by a passing apple. Fleming left the window open and came home to penicillin. Readers are asked to provide a fictitious account (short) of similar momentous discoveries which were happened upon by chance. If stuck for topics, they might try their teeth on pulsars, double helices or good old continental drift. Closes (Dec. 5). □

Science and Technology to Development (CASTARAB) in 1976, to be organised with the cooperation of ALECSO and the Economic Commission for West Asia (ECWA).

As a first step in preparation for the conference, a preparatory meeting of experts was held in Kuwait to advise the Director General of UNESCO on the preparation of the agenda and the documents for the CASTARAB conference.

It finally recommended three main themes for the conference: trends in the national science and technology policies of Arab states; regional co-operative research projects relating to natural resources, energy, food resources and environmental quality; and integration mechanisms for science and technology in the Arab states.

● Saudi Arabia has invited Egypt to make use of the Centre for Applied Geology (CAG) which had been established in Jeddah, close to the Arabian shield (which is rich in ores and minerals). The CAG is at present offering courses in mineral exploration, hydrogeology and engineering geology. It offers the degree of Master of Science and has a two-year geology technician training programme leading to a technical diploma.

The centre also offers an opportunity for geologists from Saudi Arabia and neighbouring countries to be trained, and students obtaining their master's degree with outstanding grades may be granted a government scholarship for completing their studies for a PhD abroad.

● A study on the brain drain of physicians in Egypt shows that the main concentration in the Egyptian population is in the Nile Valley and its Delta, where the limited amount of land space means that about 99% of the total manpower is crowded into about 3.5% of the total surface area of the country. Consequently there is a regular redistribution of manpower and Egyptian physicians tend to migrate (both internationally and intranationally) with the UK and the USA proving the most attractive destinations.

Apparently, African countries prefer Egyptian physicians, with the result that there is a migration of medical students from Arab and African countries to the Faculty of Medicine at Cairo University. This year the main exporting countries for medical students were Saudi Arabia (297), Libya (181), Palestine (134), Jordan (121), Sudan (114), Kuwait (102), Lebanon (88) and other Arab countries (278)—a total of 1,315 from Arab countries; they also came from South Africa (59), Malaysia (48), Nigeria (26), other African countries (41), from Pakistan (25), and other countries (54) (making a grand total of 1,568 foreign students).

Statistics show that physicians left jobs in the rural areas at the following rates: 1972 (5%), 1973 (4.2%), 1974 (5.8%); and the number of Egyptian physicians working abroad were spread in the following way: in Arab and African countries in 1965, 269 (76.3%); in African countries 23 (6.4%); and in other countries 61 (17.3%). □

Carcinogenic agent?

A SOLVENT used extensively in academic and industrial laboratories may be a potent carcinogen when its vapours are inhaled, according to tests carried out by scientists at the DuPont corporation in Wilmington, Delaware.

Known as hexamethylphosphoramide (HMPA), the solvent has long been recognised as acutely toxic and as a skin irritant, but there had been no previous indication that it is carcinogenic. The DuPont study found, however, that rats developed a rare nasal cancer when they inhaled small amounts of HMPA for 6 to 8 months. Although the study has not yet been completed, Dr J. A. Zapp, Jr, Director of DuPont's Haskell Laboratory for Toxicology and Industrial Medicine, wrote a letter to *Nature* to "urge everyone using HMPA to handle it with the precautions appropriate to a potential carcinogen".

The experiment, which was started in December last year, involved exposing rats to amounts of HMPA ranging from 0 to 4,000 parts per 10⁹ for 6 hours a day, 6 days a week—in other words for periods of time corresponding to occupational exposure. After 8 months, some of the rats developed enlarged noses and had difficulty in breathing, and it turned out that they were suffering from a form of nasal carcinoma. "There appears to be no doubt", Zapp said, "that this rare form of cancer has been produced . . . by inhalation exposure to HMPA in a concentration as low as 400 p.p.b.". □

correspondence

EEC directives

SIR,—In his article "A community of interests" (October 16), Lord Ashby describes as "phoney" the EEC argument that to permit lower effluent standards to a Scottish wood pulp mill than to one situated on the Rhine constitutes unfair competition.

The EEC argument is valid since the clear intention of the 1973 Declaration is to remove the question of pollution control from the realm in which market forces can operate. The situation is analogous to that of safety regulations inside the suggested wood pulp mills. Such regulations are recognised as imposing a prior cost on the production process. Society, through the legislative process, has opted for such. Price competition can begin after these legislated costs have been paid—and a company can only stay in business if its rivals are obliged to do the same.

B. A. MARDALL

London, UK

SIR,—Regarding Lord Ashby's article on EEC directives and pollution, there is surely now real evidence that sewage in seawater around beaches is dangerous to health. I refer to work by Dr H. Williams Smith, a bacteriologist, who analysed samples of seawater from fifteen beaches in England and Wales, and found high concentrations of *E. coli* which had clearly got there from sewage. As Dr Smith pointed out in 1971, this could lead to *E. coli* (which are themselves harmless) being swallowed while swimming and passing on the property of resistance to antibiotics to bacteria in the swimmer's gut.

JOHN NEWELL

BBC, London, UK

The science of astrology?

SIR,—Although one can deplore the mumbo-jumbo of astrology and its patently false postulate that planetary and stellar positions are relevant to the human condition, the condemnation of it in immoderate terms by 186 scientists (September 18) could well prove equally deplorable. It should be remembered that modern chemistry springs from alchemy, beside which astrology seems almost rational!

Perhaps astrology, like alchemy, represents a corpus of observational data collected over millennia which has not yet been codified. It is entirely credible

that a foetus conceived in winter will differ (on account of maternal stress from the environment) from one conceived in summer, and the difference might have behavioural implications. If so, astrology could prove to be a repository of data, access to which is attained by what is virtually an elaborate calendar.

Anthropological or social-science studies along these lines might be profitable; certainly without them outright condemnation is rash.

S. SMITH

Epsom, UK

Botanical decline

SIR,—The declining interest in the botanical sciences is causing concern to biologists, and especially plant scientists all over the world. The implications of declining interest in plants are far reaching, particularly when the need for increasing food supplies becomes more and more urgent. It is therefore of interest to see how the plant sciences are represented in a general science journal such as *Nature*. Without claiming to have made a statistically significant study, I think the following figures are striking indeed, I counted the biologically oriented Letters to *Nature* in 10 recent issues of *Nature*. In 10 issues there were 236 such communications. Of these, 14 dealt with subjects which may be regarded as botanical in the wide sense of the word. Of these 14, two dealt with photosynthesis, three with plant biochemistry and four with fungal metabolism. In other words, the entire area of plant sciences is represented by not quite 6% of the letters to *Nature*. The results of this are of course obvious. Plant scientists being aware of the few botanically oriented articles in *Nature* will respond in two ways. They will tend not to read *Nature*, a fact easily confirmed by casual consultation of colleagues. In addition they will tend not to send papers to *Nature*, because they will readily be overlooked by those most interested.

The existence of fashions in science is well known. What is perhaps not always appreciated is just how invidious the effects of such fashions are. A fashion will lead to increased publication, which in turn draws increasingly the attention of those not working on well defined, clearly oriented problems. Such new criteria as citation indexes aggravate this. One should also

not ignore the repercussions which publication policies have on the availability of research funds. The problem of fashion exists, of course, not only in general science journals but also in specialised ones. Very often the disastrous results are not due to any deliberate editorial policy but to a snowball effect. Articles in a fashionable field will lead to an increasing volume of articles in the same area. It may be assumed that the number of good articles will be greater, the great the number of articles submitted (although this will certainly not be a linear relation). It is then inevitable that articles on fashionable subjects will flood the journals and decrease the interest in less fashionable subjects.

A. M. MAYER

The Hebrew University of Jerusalem, Israel

Cats' eyes: a new twist?

SIR,—Dr Blakemore's work on the rotation of kitten's eyes has—quite rightly—raised a storm of protest and we in the RSPCA are obviously extremely concerned at any suggestion that physical or psychological suffering has been inflicted, to whatever degree.

What also concerns us is that we seem to have yet another example of unnecessary experimentation on living animals. Work by Stratton in 1897 was developed by Snyder and Pronko (1950) and others, leading to that published by Held and Baur in 1967 and subsequently.

It is now well established that no adaption occurs in the primary visual cortex when there is rotation of the visual image; and that visual perception involves the integration of both visual and non-visual activity by the higher centres.

In view of this all that Blakemore seems to have done is to have established that rotation of the retina produces similar effects to those produced by using prisms.

Why does he not admit that this was simply another ill-advised academic exercise instead of attempting 'post hoc' justifications of his 'experiments' on the ground that they will contribute to the treatment of various afflictions which are either incredibly rare, readily studied in humans or both.

D. A. PATERSON

Royal Society for the Prevention of Cruelty to Animals, Sussex, UK

news and views

A TECHNICAL Conference on Tropical Moist Forests (rainforests in the demotic) was to have been held in Brazil this September. The conference, organised by the Food and Agriculture Organization (FAO) of the United Nations, was cancelled in June 1975. With tropical rainforests everywhere under increasing pressure from human activity, this cancellation is doubly unfortunate: on a technical level it retards the dissemination of knowledge, and on a symbolic level it suggests unconcern.

Earlier ecological theory frequently held that complexity, in the sense of many species and a rich web of interlocking relationships, tended to confer stability on a community. More recent work, both empirical and theoretical, suggests that complex ecosystems (of which the rainforest is the archetype) are dynamically fragile. Although well adapted to persist in the relatively predictable environment in which they have evolved, tropical rainforests are likely to be much less resistant to the disturbances wrought by man than are relatively simple and robust temperate ecosystems. Much evidence and thinking in support of this view comes from the report of the (US) Institute of Ecology (TIE) entitled *Fragile Ecosystems: Evaluation of Research and Applications in the Neotropics* (edit. by Farnsworth and Golley, Springer, Berlin, 1974), and from the First International Congress on Ecology held in 1974 (see *Nature*, **251**, 376–377; 1974).

Such generalisations about entire ecosystems can be related to the life history strategies of the constituent species, which may usefully be discussed in terms of the deliberately oversimplified concept of *r* selection and *K* selection. This general notion goes back to Schmalhausen, Simpson, Stebbins and others, but it was MacArthur and Wilson who coined the phrase “*r* and *K* selection”, derived from the conventional parameters in the logistic equation, $dN/dt = rN(1-N/K)$. This equation describes the familiar, sigmoid curve of ultimately bounded population growth: at low population densities, there is essentially pure exponential growth, at the rate *r*; at high densities the population stabilises at a value *K* which is set by the environmental carrying capacity. A *K*-selected organism sees its environment as stable and predictable (and con-

The tropical rainforest

from Robert M. May

sequently the population is usually around its equilibrium value, $N \approx K$). The evolutionary pressures on an organism in these circumstances are, crudely, to be a good competitor; to increase its effective value of *K*; to have fewer offspring but invest more time and energy in raising them. Conversely, an *r*-selected organism sees its environment as unstable and unpredictable (and is usually at low population values, growing exponentially, and undergoing episodes of boom and bust). The evolutionary pressures here are for opportunism; for large *r* to exploit the transient good times; to have many offspring, few of which can expect to mature. Of course, reality is more complicated than this, and the paradigms of *r* and *K* selection are no more than opposite ends of what in fact is a continuum.

Notice that it is difficult to produce extinction in *r*-selected organisms, such as crop pests or the fire ant; their primary adaptation is to bouncing back from low population values. Conversely, *K*-selected organisms are geared to maintain the population around its equilibrium value, and may have difficulty in recovering from a severe disturbance. The extinct passenger pigeon is an example of a once abundant organism of a *K*-selected kind.

Broadly speaking, the contrast between the relatively predictable and stable tropical environment and the relatively vagarious temperate one can be viewed as tending to produce a *K* selection/*r* selection contrast. One manifestation of this is that ecologically analogous animals tend to give birth to fewer progeny in the tropics than in the temperate and boreal zones. Examples are agouti and paca compared with rabbits, peccaries with wild pigs, curassows and guans with pheasants and quails. Lack, Cody and others have presented a wealth of evidence that birds tend to have smaller clutch sizes in the tropics: this and other such data have been reviewed by Southwood *et al.* (*Am. Nat.*, **108**, 791–804; 1974). The overall effect is that tropical animals tend to have less capacity for the sort

of explosive, “pioneer” population growth which characterises their higher-latitude cousins (mythologised by lemmings). They are not well adapted to recovery from bad times.

There is a corresponding tendency to select for competitive ability, rather than weed-like opportunism and adaptability, in tropical plants. One consequence is that seeds tend to have little or no dormancy period, being adjusted to germination in the shady, moist, cool environment of the forest. This is in striking contrast to the wide variety of dormancy periods and survival strategies used by, say, desert plants. Thus, for example, members of the Dipterocarpaceae, the main timbertree family of South-East Asia, have seeds adapted to germinate in the stable microclimate (23–26 °C) of the rainforest soil. Clearing results in severe heating of the soil, and seedling death. The inability of these and many other seeds to recolonise, once sufficiently large areas are disturbed has led Gomez-Pompa *et al.* (*Science*, **177**, 762–765; 1972) to label the tropical rainforest a ‘nonrenewable resource’. Management of rainforests for a sustained yield timber industry is likely to require more sophisticated techniques than for temperate forests, a point underlined in the forestry conference report in *Nature* (**255**, 578; 1975).

Another relevant fact is that the sustained higher temperatures and high humidity in the tropical soil micro-environment makes for much faster decomposition rates than at higher latitudes. The typical turnover time for the decomposition of leaf litter in the tropical rainforest is 6 weeks, compared with 1 year in temperate deciduous forests, 7 years in boreal conifer forests, and 3 years in temperate grassland (Whittaker, *Communities and Ecosystems*, second ed., 248; Macmillan, New York, 1975). Tropical forests tend to respond by storing more of the nutrient pool in the plant biomass, and less in the soil, relative to temperate and boreal forests. This tends to protect lateritic tropical soils from weathering and washout of minerals and nitrates; removal of forests, as in efforts to expand agricultural production, tends to quicken laterisation, which in turn impairs agriculture.

These differences, in the tropics, between soil types which range from volcanic to lateritic, mean that un-

planned land occupation can have unfortunate outcomes. Summarising such problems in South-East Asia, Meijer (*Bioscience*, **23**, 528-533; 1973) has written: "In Vietnam, Thailand, Malaya, Sumatra, Java and the Philippines, only those areas where local agriculture is built around irrigated rice fields can have a stable agriculture and a dense population living in harmony with the environment. The vast hilly sandstone regions of Sumatra and Borneo are not suitable for continuous short-term crops, hill rice or corn; it is, in the long run, economically more productive to keep them as Forest Reserves harvested on a sustained yield basis". Eckholm's review (*Science*, **189**, 764-770; 1975) of the deterioration of mountain environments stresses the consequent effects on the lowlands, and particularly on

tropical lowlands: "The eastward movement of settlers to the jungles of the upper Amazon Basin can appropriately be labelled anarchic.—The frequent result is double disaster: a costly depletion of lumber resources as trees are cleared without regard to the soil's suitability for agriculture, followed shortly thereafter by the abandonment of the unproductive forms".

With most of the Top Ten human population growth rates currently attained in equatorial countries, an acceleration of destructive impacts on tropical rainforests is inevitable. But the long-term effect of this impact could be mitigated by coherent and well informed planning and management. It seems more likely that, in a decade or two, it will be possible to organise a retrospective conference on Tropical Moist Forests. □

Organisation of the mammalian tectum

from Shin-Ho Chung

A NUMBER of recent anatomical and physiological studies on the mammalian tectum (or superior colliculus) provide a glimpse of the ways in which the brain processes sensory inputs to make a final motor command. Axons carrying visual, auditory and somatosensory information converge on the tectum and interlace with tectal neurones, forming an intricate network of nerve cells; the main output of the tectum is then relayed to a number of motor nuclei that control the movement of the eyes or the head towards the source of stimuli. In a sense, the tectum is a microcosm of the entire brain, because it serves as a correlation centre for incoming messages and a command post for eliciting behavioural responses. For this reason, it has fascinated neurobiologists for several decades.

In the vertebrates below mammals, the tectal and subtectal areas are the main centres of termination of sensory pathways. In the course of phylogeny, the tectal areas become progressively less important, and some of their functions are assumed by the diencephalon and the cortical centres. The relative size of the tectum is reduced as the diencephalon is enlarged, and this condition becomes progressively more evident from amphibians towards birds and mammals. In all mammals, however, the tectum retains its function of controlling the reflex turning of the head or eyes towards the source of external stimuli.

From the dorsal surface, mammalian tecta appear as a pair of hemispherical bulges in front of the cerebellum. Cell bodies in the tectum are organised into distinctive laminae arranged concentrically around the central grey matter, and each cellular band is separated by a layer of axons originating from

various parts of the brain. Axons stemming directly from the eyes terminate at the most superficial layer of the tectum, with those from the contralateral eye (crossed fibres) positioning slightly more superficially than those from the ipsilateral (uncrossed fibres). Two recent anatomical studies (Graybiel, *Brain Res.*, **96**, 1; 1975; Hubel *et al.*, *ibid.*, **25**) have revealed the intricate pattern by which terminals of optic axons from the two eyes are distributed on the surface of the tectum. The pattern varies systematically as fibres enwrap the tectum from the front to the back. On the posterior half of the tectum, for instance, clusters of uncrossed fibres form regularly spaced terminal arborisations giving the appearance of a chequerboard pattern. Crossed fibres, on the other hand, are distributed as a continuous sheet overlying the terminations of the ipsilateral fibres. The upper margin of this sheet is smooth and well defined, while the lower margin shows regularly spaced indentations interdigitating with the ipsilateral terminals.

Fibres from the visual cortex, which constitute the phylogenetically new secondary visual system, terminate at a slightly deeper level of the tectum, and there link up with the older direct retino-tectal system (Kuyper and Lawrence, *Brain Res.*, **4**, 151; 1967; Kawamura *et al.*, *J. comp. Neurol.*, **158**, 339; 1974). Although the mode of synaptic terminations of the direct and indirect visual fibres on tectal cells has not been clearly elucidated, it is known that a single tectal cell sometimes receives inputs from both sources (Sterling, *Vis. Res.*, **11**, 309; 1971; Hoffman, *J. Neurophysiol.*, **36**, 409; 1973).

In addition to the visual inputs, the

tectum receives projections from other sensory modalities, mainly the auditory, proprioceptive and somatosensory systems. These inputs originate from various parts of the brain, including the inferior colliculus (Powell and Hatton, *J. comp. Neurol.*, **136**, 183; 1969), auditory cortex (Garey *et al.*, *J. Neurol. Neurosurg. Psychiatry*, **31**, 135; 1968), spinal cord (Mehler, *Ann. N. Y. Acad. Sci.*, **167**, 424; 1969) and motor cortex (Sprague, *Anat. Rec.*, **145**, 288; 1963), and terminate at the deep layers of the tectum. Tectal neurones appear to form a synaptic chain, with superficially located cells synapsing onto successively deeper ones. At each level, extrinsic afferent fibres make further synapses with this intrinsic chain of neurones (Sprague, *Neurosci. Res. Prog. Bull.*, **13**, 204; 1975). The main output of the tectum is then channelled into the two motor nuclei (the Interstitial Nucleus of Ramon y Cajal and the Nucleus of Darkshevitich) and certain parts of the spinal cord that control eye and neck movements (Altman and Carpenter, *J. comp. Neurol.*, **116**, 157; 1961).

These anatomical descriptions accord with physiological studies. Cells in the superficial layers of the tectum have small visual receptive fields, responding optimally to a moving stimulus. Other cells, probably located slightly deeper than these simple cells, show several added complexities in their response pattern. For example, they show preference for a particular direction of movement, discharging briskly only when the visual stimulus is moving in a certain direction. After destruction of the visual cortex, this feature is seldom found, thus indicating that the cortical connections are responsible for this complexity (Wickelgren and Sterling, *J. Neurophysiol.*, **32**, 16; 1969; Rosenquist and Palmer, *Expl Neurol.*, **33**, 629; 1971; Berman and Cynader, *J. Physiol., Lond.*, **245**, 261; 1975). There is a marked increase in the size of receptive fields with increasing depth in the tectum, and cells in the deep layers respond to visual as well as somatosensory and auditory stimuli (Cynader and Berman, *J. Neurophysiol.*, **35**, 187; 1972).

Not only are the inputs to the tectum organised in laminae, they are also topographically ordered with respect to the external world, so that all the cells in a given column attend to the same part of the environment. Gordon, *Science*, **173**, 69; 1971; *J. Neurophysiol.*, **36**, 157; 1973) first showed that the optimum position of a sound source relative to the animal's head, for eliciting responses from a particular part of the tectum, corresponds with the positions of visual receptive fields of cells in the same part of the tectum. Moreover, if a

visual cell shows a particular preference for a given direction of movement, the deeper tectal cell responds best to a sound source moving in that direction. The columnar overlap of different sensory modalities has recently been re-examined (Dräger and Hubel, *Nature*, **253**, 203; 1975; *J. Neurophysiol.*, **38**, 690; 1975; Stein *et al.*, *Science*, **189**, 224; 1975). In both the cat and mouse, the map of the visual space is topographically coincident with the somatosensory representation of the animal's body. In the mouse, protruding whiskers cover the major part of the visual field. A tectal cell responding to gentle tapping of a whisker lies below a visual cell looking at that whisker. In those parts of the tectum involved in the visual field where no whiskers are in the way, somatosensory responses at deep levels are elicited from other parts of the body. For instance, inferior visual fields where the mouse may see its own paw are associated with tactile fields on the paw. The output of the tectum is then somehow coupled to the motor system such that the animal brings the source of stimuli into its central visual field. This has been demonstrated by directly stimulating different parts of the tectum electrically (Schiller and Stryker, *J. Neurophysiol.*, **35**, 915; 1972).

The hierarchical organisation of nerve cells, as found in the mammalian tectum, is the general principle on which all nervous systems are constructed. At each successive level, incoming messages are further processed, and the stimulus features to which a neurone will respond become more specified. Elucidating the rules whereby important features of the external world are extracted and recombined by the nervous system has been one of the fundamental problems in neurobiology. It is toward this aim that a group of neurobiologists are gradually building up the basic 'wiring diagram' of the tectum. What developmental mechanisms ensure the formation of such an exquisitely ordered structure are likely to remain unknown for a long time. □

Host selection by a parasitic mite

from F. E. G. Cox

MANY parasites make use of chemical signals to identify suitable hosts but in few cases have either the nature or the sequence of these signals been characterised. Arthropods usually exhibit a two-stage pattern of behaviour in which they first search for their hosts and then orientate themselves with respect to chemical stimuli emanating therefrom. Most studies on host finding have been based on insects such as



A hundred years ago

WE have received an address by Prof. R. H. Thurston, C.E., delivered to the graduating class of the Stevens Institute of Technology (U.S.). It is entitled "The Mechanical Engineer, his Preparation and his Work," and contains some excellent advice, useful not only to young engineers, but to all who have been trained to other mechanical professions. The Stevens Institute, though what we would call a technical college, affords a good general scientific training, with a fair admixture of literary culture, and the object of Prof. Thurston's address is to show that the more complete is the culture of an engineer, the greater is likely to be his professional success.

from *Nature*, **13**, 16; Nov. 4, 1875

mosquitoes that attack man (see for example Gillies and Wilkes, *Nature*, **252**, 388; 1974) but in this issue of *Nature* (page 788) Egan, Barth and Hanson describe how a mite that parasitises cockroaches locates and identifies its host. These authors used simple two-choice preferential tests and the attractant substances were applied to paper disks under controlled conditions. In all, the responses of 12,000 mites were examined. In itself, this study has little practical application in the immediate future, but it opens up possibilities of analysing the factors that cause mites to attack man and his domesticated animals and the development of methods of protection against these ectoparasites and the diseases they transmit.

The mite used was *Proctolaelaps nauphoetae* which is specific to and gregarious on the cockroach *Nauphoeta cinerea*. *P. nauphoetae* is first attracted to faecal materials and the sorts of things that occur in cockroach nests, such as pieces of limbs and rotting organic material. The identification and orientation towards such materials is likely to bring the mites to a cockroach nest, and it was confirmed that they can recognise nest "markers" because they preferentially seek out individuals that have been living in colonies, in contrast to those of the same species that have been living alone, and they are also attracted to non-host species that have been placed in host colonies.

Having come into the proximity of a cockroach colony, a second level of attraction enables the mites to identify cockroaches belonging to the same family as the host and to avoid others. The attractant consists of the expectorants which are spread over the body during grooming. The mite has then

to determine whether or not the cockroach is the appropriate host species. The actual substance that is used was isolated by placing paper disks in the colony and extracting the attractant from them. The authors tentatively identify this substance as a poly-amino sugar and call it "nauphoetamine". The identification of "nauphoetamine" by the mite is the final stage of the host-finding behaviour but it is important to note that chemical stimuli are involved in the first two stages: the recognition of the proximity of any kind of colonial cockroaches by faecal and detritus stimuli, and direct orientation to expectorants which brings the mite to a particular group of cockroaches which might or might not be the correct host species.

The fact that the mites show three separate levels of discrimination and that one at least of the attractants can be isolated means that it should be possible to attract mites away from potential hosts or towards pest species if biological control is the aim. Even if these studies do not lead to any further progress in the manipulation of mites they will be of general interest to parasitologists in several areas where host finding and identification are poorly understood and at present, in the case of warm blooded animals, often simply attributed to host temperature. □

Is submicrominiature small enough?

from Andrew Holmes-Siedle

SOLID-state science has made it possible to put all the circuits of a computer onto a piece of silicon no larger than a postage stamp (say 1 cm² in area). It is reasonable to ask how much further one should try to go. Should we now put the computer onto a pinhead or put a faster, more versatile computer with much more storage onto the same square centimetre? The idea has its attractions because we are never short of information to process (visual information flows in on us at more than 10 million bits per second, for example), and it saves energy if we cut down the amount of purified electronic material needed to make the circuits.

Two authoritative surveys on the ultimate limits to the density of electronic functions on a wafer of electronic material have appeared recently. One, by J. T. Wallmark (*Institute of Physics Conf. Ser.* No. 25, 133; 1975), starts from the practical necessities of integrated-circuit technology and, through original calculations, reaches quantitative conclusions. The other, by R. W. Keyes of IBM Research Centre (*Proc. IEEE*, **63**, 740; 1975) starts from some basic laws of device physics, collects results of many other workers and produces a set of conceptual tools rather

Comparison of physical limits in digital electronics

	Mathematical symbol	Units	Typical 1965 circuit	Typical 1975 circuit	Wallmark's ultimate circuit	Ultimate if pattern generation were only factor	Human brain
Number of transistors or other active elements	N		10	10^4	10^5	2.5×10^9	10^{10}
Linear dimension of chip	$\sqrt{(A_c)}$	μm	6.6×10^3 (0.66 cm)	10^4 (1 cm)	6.6×10^3 (0.66 cm)	10^4 (1 cm)	—
Area of chip	A_c	μm^2	4×10^7	10^8	4×10^7	10^8	—
Area per active element with allowance for interconnections	A_c/N	μm^2	4×10^6	10^4	4×10^2	4×10^{-2}	—
Side of active element (e.g. whole transistors)	$\sqrt{(A_c/N)}$	μm	2×10^3	10^2	20	2×10^{-1}	4
Linewidth, d , required to fabricate element	$10^{-1}\sqrt{(A_c/N)}$	μm	200	10	2	2×10^{-2}	—
Minimum edge uncertainty, ΔL , for above linewidth	$5 \times 10^{-4}\sqrt{(A_c/N)}$	μm	1	5×10^{-2}	10^{-2}	10^{-4}	—
Wavelength of light required	$2\Delta L$	μm	2	0.1	2×10^{-2}	—	—
Energy of electron beam required	$2h^2/m\Delta L^2$	eV	≤ 1	< 1	~ 0.1	100	—

than quantitative conclusions. The two are thus complementary, and provide a good foundation for the statement that we cannot much longer continue the trend by which the density of devices per unit area is doubled annually. Physical laws will in fact soon prevent us. The limiting processes include the propagation of light, thermal conduction, electromigration and dielectric breakdown. The first three columns of the table, derived loosely from Wallmark's and Keyes' formulae, show where the downward trend in size must stop. While, in the past ten years, the area of an active, multielement structure such as a transistor has gone down from about 1 mm^2 to 0.01 mm^2 , giving a minimum element width, d , of $10 \mu\text{m}$, Wallmark gives good arguments as to why it can never go lower than $2 \mu\text{m}$. One major problem, very difficult to circumvent, is that the local concentration of doping impurity atoms fluctuates noticeably on this scale so that small elements such as resistors, intended to be of the same value, will in fact fluctuate in value. Wallmark is alone in seeing this as the main physical barrier, but both authors find that active devices, such as transistors can only pass a certain current density before breakdown phenomena occur, and the size limit for this effect again occurs when d is a few micrometres.

It is worth noting that the technology for making linewidths much smaller than $2 \mu\text{m}$ is well in hand. While conventional ultraviolet light cannot be used near Wallmark's limit (see table), the wavelengths of low-energy electron beams are such that much smaller patterns could be made with ease. I would estimate that, if pattern generating were the only limit, one could fit more than 10^9 transistors on a chip of area 1 cm^2 (see fourth column of table), but electrical breakdown, heating and electromigration effects would attend the high current densities and electric fields which these much smaller elements would experience. The reason why fields increase as size decreases stems from the existence of voltage noise. Random voltage fluctuations of value kT (0.025 eV at 300 K) occur across

junctions and the applied voltages must swamp these. Thus, voltage cannot be scaled down with element size. Keyes further insists that, with all digital logic devices, the mode of operation must be such that large non-linearities in the I - V characteristics are involved. This requires operating voltages of 10 – 100 times kT . In non-digital systems, however, smaller energy transfers can possibly be used to process information. Keyes gives an interesting study of the bare essentials of a logic operation, namely two potential wells with a small barrier between them, the barrier being of the order of kT . In the ideal case, the energy dissipated in one operation of this system is of the order 10^{-21} J at 300 K and this is the quantum limit. By contrast, low-power logic uses about 10^{-10} J per operation. The only known information-processing system which approaches this efficiency is a natural one—the passage of information during nucleic acid transcription, which involves about 10^{-19} J per operation. The advantage which nature has here is that speed of processing is of little importance to cell growth. The human brain, requiring greater speed of operation, operates at about the same energy per operation as low-power electronic logic.

A difference in conclusions on minimum size, as reached by Keyes, Wallmark and another earlier study (Honeisen and Mead, *Solid St. Electron.*, **15**, 819, 891; 1972) lies mainly in their ideas of which elements of the circuit will in future be the controlling ones. For this, Keyes chooses the interconnections between devices, in which the speed of light will limit speed of transport and high current densities can produce heating and electromigration (minimum linewidth, $10^{-1} \mu\text{m}$). Honeisen and Mead see dielectric breakdown as an earlier barrier (minimum width, $1 \mu\text{m}$) while, as mentioned, Wallmark takes the broader view and considers that the statistics of pattern generation (not fully considered by the others) and doping fluctuations will raise the limiting width well above $1 \mu\text{m}$. Agreement on the minimum power dissipated for a given speed of

operation is fairly good. At room temperature, voltage fluctuations and the capacitances of components will not allow speeds of operation faster than a few tenths of a nanosecond or power levels below a nanowatt per element. In special cases, cooling to cryogenic temperatures can lead to lower power levels, although commonsense suggests that almost all the uses for the most minute logic devices will tolerate neither the inconvenience nor the power drain involved in continuous cooling. It seems that, unless we can develop some very unusual electronic materials, the only way to encompass extremely small size in computers is to accept a slower-than-normal rate of operation. Furthermore, we might as well accept that we will never get the equivalent of a videotape recorder or Atlas computer which will fit comfortably in the pocket.

Hormonal regulation of hormone receptors

from J. R. Tata

THE central issue in research on hormone action is a better understanding of hormone-receptor interaction. Until recently, kinetic analyses of hormone receptor function have been based on the assumption that the number of receptor sites per target cell is constant, or that its fluctuations are of little physiological consequence, so that what really matters is the number of hormone molecules that are available for interaction with receptor sites. Reports are, however, now trickling in to suggest that the number of receptors per cell can vary sufficiently to influence the response of the target cell to the hormone. What renders this idea particularly intriguing is the possibility that a hormone can modulate the cellular level of its own receptor or that of another hormone.

Hinkle and Tashjian, Jr. (*Biochemistry*, **14**, 3845; 1975) have recently examined the effect of thyrotropin-releasing hormone (TRH), a hypothalamic tripeptide, on the number of

receptors for this hormone in a clonal strain of rat pituitary tumour cells (GH₃ cells). These cells do not make thyrotropin (TSH) but TRH does stimulate the synthesis and release of the hormone prolactin and inhibits the formation of growth hormone by these cells. Previously it had been shown that TRH bound firmly to GH₃ cells, presumably to receptors on plasma membranes of the type found in normal pituitaries (Vale *et al.*, *Endocrinology*, **93**, 26; 1973; Gourdji, *et al.*, *Expl Cell Res.*, **82**, 39; 1973) and that the binding preceded the action of the hormone. According to Hinkle and Tashjian, incubation of GH₃ cells for 2–3 days with TRH caused a reduction in the number of binding sites for TRH to a third of the normal level. Receptor molecules seem to be long-lived and their loss itself requires active protein synthesis since cycloheximide protects the cells against receptor depletion. A consequence of such a hormone-induced loss in receptor capacity would be that it would provide a target cell with the means to regulate its sensitivity to the hormone.

Is the above example of a hormone regulating the number of its own receptor molecules a peculiarity of abnormal tumour cells or of culture conditions, or does it reflect a universal physiological homeostatic mechanism for hormones? After all, GH₃ cells do not synthesise TSH whereas the action of TRH on normal pituitary cells is to control their TSH output. In another pituitary tumour cell line, GH₁ cells, whose growth is influenced by thyroid hormone, Samuels and Tsai reported that tri-iodothyronine (T₃) increased the number of T₃ receptors in the nucleus (*Proc. natn. Acad. Sci. U.S.A.*, **70**, 3488; 1973). But, Spindler *et al.* (*J. biol. Chem.*, **250**, 4113; 1975) have failed to observe a similar phenomenon for the number of T₃ receptors in normal rat liver nuclei. It may, therefore, be premature to generalise about the self-regulation of hormone receptors. Perhaps one needs to examine those systems where sensitivity of a tissue to a hormone changes very rapidly, as during ontogenesis or during certain hormone-dependent cyclical physiological situations. It may well be that the basis of the "positive feedback" phenomenon during amphibian metamorphosis may be due to marked changes in the number of T₃ receptors in the tadpole hypothalamus. Similarly, oestrogenic hormones regulating the level of their own receptors may underlie the cyclical variations in the hormonal sensitivity of the uterus during the oestrus cycle. In this context, an interesting example of induction of oestrogen receptor by oestradiol was reported by Gschwendt and Kittstein (*Biochim. biophys. Acta*, **361**, 84;

Eruption anticipated

from Peter J. Smith

ON July 5–6 this year the Hawaiian shield volcano, Mauna Loa, erupted for the first time in 25 years. The renewed activity was not entirely unexpected, however, for only a few weeks before the eruption scientists at the US Geological Survey's Hawaiian Volcano Observatory had prepared a report, based on seismic and geodetic data, suggesting that Mauna Loa "may be reawakening". As it turned out, the report was overtaken in press by the event, but now that it has been published (Koyanagi *et al.*, *Geophys. Res. Lett.*, **2**, 405; 1975) it is possible to see the evidence upon which the prediction was based.

Since Mauna Loa last erupted in 1950, seismic activity in its vicinity has been low but not completely absent. Records covering the past 13 years show that there were several bursts of activity which reached small peaks in 1962, 1967 and 1970, each larger than the one before. But in April 1974 the frequency of small summit earthquakes increased con-

spicuously, reaching hundreds to thousands a day for a week in mid-August, more than a week in mid-December and for many days between February and June 1975. Throughout the whole period 1962–1975 magnitudes ranged up to 4.6 but most of the events with magnitudes greater than 3 occurred during 1974–75.

During the latter period the shocks generally became not only larger but also shallower than before. Most of the foci were at depths of less than 15 km, although a few reached 50 km. Moreover, there was an earthquake-free zone at depths of 15–30 km beneath the summit crater, believed to indicate a magma storage region. The epicentres defined a broad seismic zone around the summit where dilation was evidently also occurring. Thus geodimeter measurements showed that a 2.5 km line across the summit caldera expanded by 10 cm between August 1974 and June 1975.

Taken together, these data strongly indicated a resumption of volcanic activity, although they were far from sufficient to enable the time of any eruption to be predicted.

receptor levels. Whatever the outcome of such efforts, the important issue will still be the perennial question of how does one equate hormone binding with the site, or sites, of relevant physiological actions of a given hormone.

A most Friendly meeting

from Natalie Teich and Paul Harrison

The Friend Cell Workshop was held at Schloss Reisenburg, West Germany, on August 28–September 1.

THE workshop established the relevance of the Friend virus–Friend cell system as a model for erythropoietic differentiation, for multiple host gene control of erythroblastic disease and for studies of the relationship between cell transformation and abnormal differentiation.

The cell biology of the Friend cell system was a major topic. Friend virus (FV) infection of susceptible mice results in enhanced erythroblastosis and polycythemia or anaemia (the original virus strain of Dr C. Friend) due to massive cell death in the early erythroblast compartment (C. Jasmin, Hôpital Paul Brousse, Villejuif). It now seems established that the target cell for FV is a committed erythroid stem cell (CFU-E) (P. Tambourin, Radium Institute, Paris). Axelrad's group (Uni-

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versity of Toronto) have isolated a strain of virus able to transform normal CFU-Es *in vitro* as judged by their ability to differentiate without erythropoietin. This may be analogous to the *in vitro* transformation of avian bone marrow cells by avian erythroblastosis virus (AEV) (T. Graf, Max-Planck-Institut, Tübingen).

A diversity of studies have evolved from the isolation of Friend erythroleukaemic cells (FLC) *in vitro* (C. Friend, Mount Sinai School of Medicine, New York). These cells are CFU-Es arrested at the proerythroblast stage, which after treatment with dimethylsulphoxide (DMSO) synthesise haemoglobin and differentiate to orthochromatic erythroblasts and possibly further (Y. Ikawa, Cancer Research Foundation, Tokyo). Other erythroid changes follow: spectrin formation and loss of H-2 surface antigen (H. Eisen, University of Geneva) and accumulation of carbonic anhydrase (F. Ruddle, Yale University). New compounds, such as highly polar molecules and fatty acids (R. Rifkind and P. Marks, Columbia University, New York) also act as inducers; haem also can induce globin synthesis and may act synergistically with DMSO (J. Ross, McArdle Laboratory, Wisconsin; A. Beudet, Baylor College, Houston). This induction of erythroid differentiation in FLC contrasts with the situation in AEV-transformed avian erythroid cells which do not differentiate or produce haemoglobin in response to classical inducers of FLC (Graf), although they accumulate globin RNA in the nucleus (A. Therwath, University of Paris).

Little is known concerning the mechanisms of action of the various inducing agents. Membrane function may be important, however. A. Bernstein (Ontario Cancer Institute, Toronto) found that local anaesthetics which increase membrane fluidity block haemoglobin production. This is consistent with other membrane changes including rapid immunological "capping" (N. Furusawa, Osaka City University), increased agglutination with plant lectins (Eisen), decreased permeability to small molecules (S. Dube, California Institute of Technology), increased anisotropy (D. Jovin, Max-Planck-Institut, Göttingen) and changes in cyclic nucleotides (R. Kram and P. Malpoix, University of Brussels).

Regarding the cell cycle dependence of the induction process, haemoglobin is not detectable until cells have been exposed to DMSO for about two cell cycles (J. Papaconstantinou, Oak Ridge National Laboratory; Marks and Rifkind). However, J. Pragnell (Beatson Institute, Glasgow) and W. Ostertag (Max-Planck-Institut, Göttingen) reported that one FLC line shows

a 5-fold increase in globin mRNA within the first cell doubling.

Several laboratories have attempted to elucidate the mechanisms regulating globin gene expression in FLC, exploiting DNA complementary to globin mRNA to measure synthesis and accumulation of globin-specific RNA. H. Aviv (Weizmann Institute, Rehovoth) stressed that relative rates of degradation of globin and total mRNAs are important in understanding how globin mRNAs predominate in the reticulocyte or differentiated FLC. Unfortunately even the sensitive techniques used are insufficient to permit similar analyses at the nuclear level to clarify the role of transcriptional and post-transcriptional regulation mechanisms. The fact that the base-sequence complexity of poly(A)-containing nuclear RNA is considerably greater than that of polysomal poly(A)-containing RNA indicates post-transcriptional control of gene activity (M. Birnie and J. Paul, Beatson Institute, Glasgow).

Another approach to regulatory mechanisms was described by P. Harrison and D. Conkie (Beatson Institute) utilising non-inducible FLC variants. In some, non-inducibility of globin synthesis is due to a dominant

defect at the nuclear level (either in globin gene transcription or an unstable primary RNA transcript); whereas in another, treatment with haem enhances globin and globin mRNA accumulation. Interestingly, in a lymphoma $\times$ FLC hybrid, globin mRNA accumulates, but globin chains do not; nor does erythroid maturation occur unless the hybrid cells are treated with haem in addition to DMSO. A regulatory role for haem in differentiation of FLC is also implied by the report of G. Kramer and B. Hardesty (University of Austin, Texas) that there are two inhibitors of translation in Friend cells, one of which is present in DMSO-treated cells only and bears close similarity to the classical haemin-controlled repressor present in reticulocytes.

Others are also using cell fusion to analyse the regulation of differentiation. B. Alter (Childrens Hospital, Boston) showed that some human globins were synthesised in heterokaryons between tetraploid FLC and diploid human amniotic fibroblasts but not in hybrids with diploid FLC. A. Skoultchi (Albert Einstein, New York) observed that hybrids between FLC and mouse hepatoma cells continue to synthesise albumin and transferrin but cannot be induced to synthesise haemoglobin or globin mRNAs. The potential of somatic cell genetics for genetic mapping was illustrated by A. Deisseroth (National Institutes of Health) by exploiting stable mouse-human hybrid clones to correlate loss of human globin genes with loss of specific chromosomes. It should only be a matter of months before the location of the α and β genes are known with certainty.

As regards the virus-host interactions, it is clear that at least three genes of the mouse determine the course of the erythroblastic disease induced by FV. The *Fv-1* locus controls the ability of the helper murine leukaemia virus (MLV) component to initiate productive infection. A second gene, *Fv-2*, controls the animal's response to the spleen focus-forming element (SFFV) of FV; in a resistant mouse, MLV and SFFV replicate and although morphologically distinct spleen foci do not appear, splenomegaly occurs later (5 weeks) (T. Odaka, Institute of Medical Science, Tokyo). K. Blank (Albert Einstein, New York) showed the restrictive effect of each locus not only on the spleen focus formation but also on the ability of the animal to limit growth of the tumour colony-forming cells. A third locus, the major histocompatibility locus (*H-2*), seems to affect not the initial infection by FV, but rather the subsequent ability to support chronic infection (H. Freedman, Albert Einstein,

Chemical and biological reactions

IN my recent comments (*Nature*, **256**, 693; 1975) on dynamics of chemical and biological reactions I mentioned work by Uzgiris and Golibersuch (*Phys. Rev. Lett.*, **32**, 37; 1974) attributing a decay term independent of scattering angle in scattered-light intensity fluctuations from haemoglobin solutions to the haemoglobin association-dissociation reaction. A paper by Haas, Mustacich, Smith and Ware (*Biochem. biophys. Res. Commun.*, **59**, 174; 1974) which disputes these findings has now come to my attention. These authors attribute the "anomalous" positive intercepts in the plot of linewidth *versus* the square of the scattering vector reported by other authors to the coincidence of the Helium-Neon wavelength and the 630 nm absorption of methaemoglobin. I am bringing these findings to the attention of your readers in view of the importance of establishing whether reaction rate of fast chemical and biological reaction can be derived from time autocorrelation functions.

I would also like to state that the fluorescence of ethidium bromide is strongly enhanced, and not quenched (as mentioned in my comments) upon binding to DNA.

H. EISENBERG

New York). G. Hunsmann (Tumor Immunology, Freiburg) reported that FLC express viral surface antigens gp71 and p12; further, immunisation either with gp71 or antiserum to gp71 will protect animals from FV disease.

Ostertag showed that treatment of FLC with BUdR and DMSO often leads to the induction of endogenous viral genomes, thus complicating the host range patterns of the Friend virus complex. Pragnell reported that sequences present in Friend virus released from DMSO-treated FLC are integrated 5–10 times in Friend cell DNA; whereas about half of these sequences (SFFV?) are absent in mouse embryo DNA. Ostertag and P. Swetley (Boehringer Institute, Vienna), as well as Ruddle, have shown that interferon treatment will effectively block virus replication without inhibiting haemoglobin synthesis. Other agents such as azidothymidine act similarly. Ostertag does, however, find a correlation between induction of differentiation and increase in intracisternal A-type particles.

N. Kluge (Max-Planck-Institut, Göttingen) and Sugiyama have established several rat erythroleukaemia lines by treating animals with dimethyl benzanthracene; these lines are induced for haemoglobin synthesis with DMSO and for virus production with BUdR. B. Weimann's (Immunology Institute, Basel) human polycythemia vera lines are not responsive to erythropoietin, but A-type particles are seen after treatment with azidothymidine. Thus, the Friend cell phenomenon is not restricted to the mouse. □

Man-made earthquakes

from E. Nyland and D. I. Gough

The first International Symposium on Induced Seismicity (ISIS) was held in Banff, Alberta, Canada, from September 15 to 19. ISIS was organised as a consequence of the Conference on Seismic Effects of Reservoir Impounding held in London in 1973, and both were instigated and supported by a working group of UNESCO. ISIS was additionally supported by the Government of Canada and was organised from the Universities of Alberta and British Columbia. The Proceedings of ISIS will appear late in 1976 in *Engineering Geology*.

AMONG engineering activities which have inadvertently produced earthquakes are the building of dams which

form large reservoirs, the injection of water into crustal rocks for waste disposal or oil extraction, and deep mining. All three topics were actively discussed, although the bulk of the papers were concerned with reservoir-triggered earthquakes.

D. W. Simpson (Lamont Doherty Geological Observatory, New York) led the papers on this topic with a comprehensive review of large reservoirs, only a small fraction of which are known to have triggered seismic activity. Among the contributed papers was one by O. V. Soboleva and colleagues (Academy of Science, Padzhik SSR), presented by Simpson who is visiting Dushanbe, USSR on a US/USSR collaborative project on detailed studies of seismicity induced by Nurek Reservoir, formed by the world's highest earth-fill dam. Epicentres migrated to the southwest end of the lake from a distance of 8 km over the years 1971–74. Focal mechanisms are complex. Six seismologists and engineers from the People's Republic of China reported on admirably thorough studies of the intense seismic activity induced by the Hsinfengkiang reservoir, where some quarter million seismic events have been detected since the lake began to fill. At least four mechanisms have been involved: initial normal faulting may have been load-induced and facilitated entry of water to deeper foci for larger subsequent strike-slip events. Another major field study, this time of the Tarbela dam in a region of very high natural seismicity in the Pakistani Himalayan foothills, was reported by Klaus Jacob (Lamont Doherty). Current discussions of earthquake control by fluid injection lend interest to a paper by C. G. Bufe (US Geological Survey) reporting a gap in seismicity on the Calaveras Fault in California: the gap is near the small Anderson Reservoir and elsewhere there are many events along the fault.

A few generalisations can be stated. Initial stress is clearly a principal factor in the triggering of earthquakes by reservoirs, but techniques for its measurement remain costly and of doubtful generality. They are almost never applied except near the dam itself. Fluid pressure is probably the main triggering mechanism, although incremental solid stress can serve where normal faults are critically prestressed. In the largest earthquakes triggered by reservoirs (Kremasta, Kariba, Koyna, Hsinfengkiang) magnitudes of mainshocks do not exceed 6.3, an empirical observation which may be related to the rock volume affected by very large reservoirs.

Carl Kislinger (Cooperatives Institute Research in Environmental Sciences, Boulder, Colorado) gave a review of theory of induced seismicity, which

finds its basis in the principle of effective stress in a porous water-filled medium and Coulomb-Mohr shear failure criteria. The extensions of these simple notions to real permeable formations has become immensely complicated. The following session showed encouraging moves toward an attack upon calculation of stress fields in porous, fractured media. Tidal triggering seems possible under at least one reservoir.

A session on mechanical and hydraulic properties of rocks related to induced seismicity was led by P. A. Witherspoon (University of California, Berkeley) with a survey of a vast body of results from his laboratory. Contributed papers considered properties of fractures, friction and sliding in rocks.

Seismicity associated with deep mining differs from induced seismicity of other types in that the excavation can itself produce stress-differences sufficient for failure, so that the effect need not, in all cases, be a triggering: initial stress may thus be lithostatic. Another special feature of this class of event is that the focal region can be visited and studied before and after the shock. A review of many years' study in Witwatersrand gold mines was given by N. G. W. Cook (Chamber Mines, South Africa). A feature of work in the central Witwatersrand is the extreme dryness of the rock there. Contributed papers included discussions of seisms produced by longwall coal mining in the United States and of seismic efficiency of mine tremors in the Witwatersrand. An earthquake sequence triggered by unloading of rock from a quarry in the Hudson valley is really explained in terms of thrust-faulting mechanisms and reduction of the vertical (least principal) stress.

A swarm of earthquakes about 10 km from a reservoir several years old, near Oroville, California, is following a mainshock on August 1, 1975. The timing was excellent in relation to ISIS, and an evening session was organised by Lloyd Cluff (Woodward-Clyde Consultants, Oakland, California). Studies by his consultant group, by the US Geological Survey and by the University of Nevada contributed to a fascinating case-history but leave unanswered the question whether the reservoir induced the earthquakes, which occurred on an active fault.

J. H. Healy (US Geological Survey) led a session on seismicity associated with fluid injection with a lucid review of results from the US Geological Survey investigations into the Denver earthquakes and the Rangely Oilfield earthquakes, both in Colorado. His was perhaps the most up-to-date review, being completed on the morning of the

session. In contributed papers there were discussions of seismicity related to hydraulic mining in New York state and seismicity associated with a complex of oil, water and gas at different levels under Lacq, France.

In seismicity related to fluid injection, fluid pressure is clearly the trigger and as with reservoirs, the pre-existing fractures and stresses are all-important and in general only become known if and when earthquakes occur. Pulling the trigger is not the best way to find out whether a gun is loaded; measurement of initial stress near future reservoirs and near oilfields where high-pressure injection is proposed could be a useful exercise for government agencies.

A session on instrumentation for observation of induced seismicity was led by a review by E. R. Kanasewich (University of Alberta), who made a strong case for introduction of digital recording systems employing miniaturised solid-state electronics and dedicated minicomputers. This triggered a brisk discussion in which the merits of many simple instruments were urged by others, particularly in adverse environments far from shop support.

The six review papers and over fifty contributed papers provoked lively and absorbing discussions and it is a matter of regret that more seismologists, and especially more engineers, did not attend. For those who did, the week seems to have been well spent. □

Growth hormone at Milan

from Mike Wallis

The Third International Symposium on Growth Hormone and Related Peptides was held in Milan on September 17–20. Abstracts have been published (*Ricerca Scientifica ed Educazione Permanente*, 2, Suppl. 1) and the invited lectures will be published in full by Excerpta Medica in 1976.

PROTEINS come in families, and this conference covered all the members of the pituitary growth hormone family, including prolactin and placental lactogen. The ground covered was thus broad (over 160 papers) and attention here will be concentrated on growth hormone (GH).

A topic which raised much discussion was the possibility that pituitary GH itself is in fact a prohormone. S. Ellis and R. E. Grindeland (NASA-Ames Research Centre, California) described studies on the nature of GH in human

plasma, and concluded that the circulating form has enhanced biological activity and reduced immunological activity compared with GH extracted from pituitaries; purification of GH from plasma is underway. U. J. Lewis *et al.* (Scripps Clinic, La Jolla) have detected various forms of GH (apparently produced by limited proteolytic cleavage) with enhanced biological activities; these are obviously candidates for the 'activated' form which appears to circulate in the blood. M. A. Vodian and C. S. Nicoll (University of California) found that bioassay and immunoassay gave widely differing results for circulating GH in the rat (and for GH secreted by pituitaries incubated *in vitro*) although the two assays agree well when used to measure the GH content in the pituitary of resting, untreated rats.

The suggestion that GH is modified *in vivo* accords well with observations that active fragments can be produced by limited proteolysis of the hormone *in vitro*. C. H. Li and T. A. Bewley (University of California) and J. L. Kostyo *et al.* (Emory University) described the characterisation of peptides from plasmin digests of GH which retain biological activity in several different assay systems. L. Graf *et al.* (Research Institute for Pharmaceutical Chemistry, Budapest) described a similar approach using thrombin. Active fragments have also been synthesised chemically, as described by F. Chillemi *et al.* (University of Milan). How these studies on active fragments produced *in vitro* can be related to those produced *in vivo* is not yet clear. One attempt to make the link has been made by J. Bornstein (Monash University) who has isolated (from pituitaries) small peptides apparently derived from growth hormone. These have been synthesised chemically, and have been shown to be active in a range of pharmacological and biochemical test systems, but how they relate to the other work on GH fragments, or to the major biological actions of GH, remains uncertain.

Biosynthetic precursors of GH have been suggested on several occasions, and a paper by F. C. Bancroft (Columbia University) provided an elegant demonstration of the probable existence of such a precursor. Messenger RNA (from a line of pituitary tumour cells) was prepared and translated in a cell-free system from wheat germ. A major product possessed immunological and chemical resemblance to GH, but was 20% larger than the normal hormone. It appears that the wheat germ system (unlike mammalian cell-free systems) lacks the enzymes which process the GH-precursor (pro-GH or perhaps pre-GH); a similar situation has been demonstrated for several other

polypeptide hormones. The existence of a precursor was also proposed by M. Wallis (University of Sussex) to explain the N-terminal heterogeneity seen in bovine GH.

A major topic of discussion with regard to the biological actions of GH was the role of somatomedins (GH-dependent peptides found in serum). It is not yet clear whether these mediate all the actions of GH, including growth promotion, or whether GH possesses some direct metabolic activity in addition to its role in promoting somatomedin production. Exciting progress in the somatomedin field was described, though it is unfortunate that some work is shrouded in secrecy as a result of the interest of the pharmaceutical industry. At least three somatomedins have now been recognised, distinguished by their relative potencies in different assay systems. Isolation and characterisation of somatomedins A and B was described by L. Frykland and H. Sievertsson (AB KABI, Stockholm). They are moderate-sized peptides, structurally quite different, and neither is a fragment of GH. K. Hall and K. Takano (Karolinska Hospital, Stockholm) described their radio-receptor assays and immunoassays for somatomedins A and B. Levels in normal human serum are quite high (about $2 \mu\text{g ml}^{-1}$ for somatomedin A, $10 \mu\text{g ml}^{-1}$ for somatomedin B). As expected, levels rise in acromegaly and fall in hypopituitary dwarfism. The somatomedins are not completely absent in the latter, however, so these factors are perhaps only partially GH dependent. L. E. Underwood *et al.* (University of North Carolina) described work on somatomedin C (a moderate-sized, basic peptide) and showed that receptors for this occur in most tissues.

Lactogenic hormones can be mentioned only briefly here. R. E. Fellows (Duke University) has characterised ruminant placental lactogens and preliminary structural studies suggest that, as has been predicted, these differ substantially from human placental lactogen.

The idea of holding a conference in which all members of the GH-prolactin family of proteins were considered was an excellent one. Although the growth hormones and lactogenic hormones are clearly related structurally, and must have had a common evolutionary origin, little resemblance has been detected at the biological level. It seems likely that structural resemblances are paralleled by resemblances in biochemical mode of action but these have not yet been clearly established. Perhaps this will be one of the themes of the 4th International Symposium on GH in four years' time. □

review article

Lateral heterogeneity and mantle dynamics

Thomas H. Jordan*

Recent work on the nature of the Earth's lateral heterogeneities yields two conclusions with implications for mantle dynamics: seismic velocity differences between continents and oceans extend to depths exceeding 400 km, and strong lateral velocity gradients at depths greater than 800 km characterise the mantle beneath many subduction zones. These results suggest that more than just the crust and upper mantle are involved in whatever form of convection is responsible for plate motions.

THE study of seismic wave velocities provides the most detailed information about the structure of the Earth's interior and its internal variation of material properties. Until quite recently, it was the consensus among seismologists that below the uppermost mantle the velocity distributions show little geographic variation. With the collection of better data and the application of high-resolution techniques during the past decade, however, it has become clear that this view cannot be maintained. Evidence is rapidly accumulating which indicates the existence of significant lateral heterogeneity throughout the Earth's mantle, even at great depths. Here I attempt to synthesise some of the evidence. Particular attention will be focused on two results which constrain the nature of mantle dynamics.

Continent-ocean heterogeneity

The extreme geographical variations in upper mantle velocities are evidenced by the large-magnitude differences in station anomalies¹ and in regional surface-wave dispersion curves². The available seismic data suggest that the large scale lateral heterogeneity of the upper mantle is largely a consequence of the structural differences between continents and oceans.

Because of the dearth of seismic recording stations in oceanic regions, it is difficult to probe oceanic upper mantle structure using conventional body-wave methods, and most of our information on oceanic structure has come from the study of surface-wave dispersion. Dispersion studies using Love and Rayleigh waves reveal that continental and oceanic surface-wave phase velocities differ out to periods exceeding 300 s (refs 3–6), and from the analysis of these data a reasonably clear picture of the structural differences in the upper 400 km of the mantle has emerged: below a depth of 50 km or so the upper mantle beneath oceans is characterised by a well developed shear-wave low velocity zone (with contrasts as high as 0.5 km s^{-1}), whereas beneath stable continental shields this low velocity zone is weakly

expressed and in some regions it may be nonexistent. Since the shear-wave low velocity zone is associated with a zone of partial melting⁷, the simplest explanation of this difference seems to be a depression of the isotherms beneath the continents, an interpretation that is in accord with modern thermal models.

But this raises a critical question with implications for mantle dynamics: what is the maximum depth to which these continent-ocean differences persist? The popular consensus is that continental plates are on the order of 200 km thick (or less) and that below this depth the sub-continental and sub-oceanic mantles are similar in composition and state^{8,9}. If this model of thin lithospheric plates is correct, then, below a depth of 200 km or so, we should observe very little structural contrast between continents and oceans.

It was first thought that the observed differences in the surface-wave dispersion data required continent-ocean heterogeneity as deep as 500 km, and this evidence was used to argue against the continental drift hypothesis¹⁰. Dziewon-ski¹¹ has demonstrated, however, that the phase velocity differences may be explained by models with the significant velocity contrasts confined above 200 km depth, which is consistent with the thin-plate concept of having continent-ocean differences concentrated in and above the low-velocity zone.

Although this conclusion is compatible with current concepts, it requires substantial modification^{12,13,18}. Evidence for continent-ocean heterogeneity below the low velocity zone has come from the inversion of free oscillation data and from recent work with shear-wave travel times.

When travel times through the upper mantle beneath the continents are compared with those predicted by the free oscillation models, surprising differences are found: travel times for the average Earth are considerably larger than expected. As shown in Fig. 1, the one-way vertical shear-wave travel times through the upper 700 km of the Earth computed from the free oscillation models (for example UTD124A or B1) are 2–3 s greater than those obtained by the direct measurement of travel times at continental-based seismic recording stations (for example the Jeffreys-Bullen Tables or SLUTD1). Jordan and Anderson¹⁹ have postulated that this baseline difference is due to continent-ocean heterogeneity. Because the eigenperiod data yield estimates of the spherically averaged velocities and because the Earth's surface is two-thirds ocean, this hypothesis requires

*Address: Department of Geological and Geophysical Sciences, Princeton University, Princeton, New Jersey 08540; Present address: Scripps Institution of Oceanography, La Jolla, California 92037.

that the average one-way travel-time difference between continents and oceans be 3–5 s for shear waves. This compares with a difference of only 1–2 s predicted by regionalised Earth models with heterogeneity confined above 400 km (for example models O1 and S1 in Fig. 1, and ref. 17). The implication is that continent–ocean differences extend below this depth.

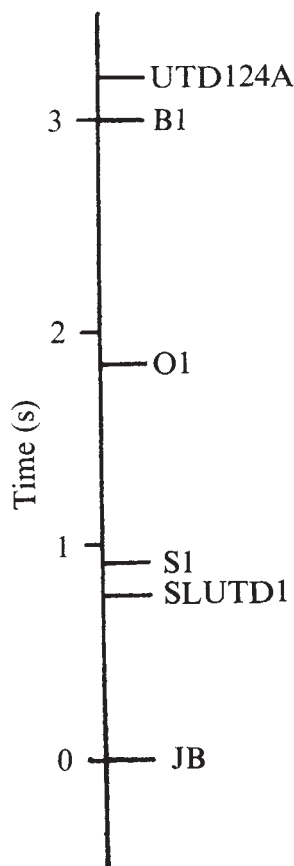


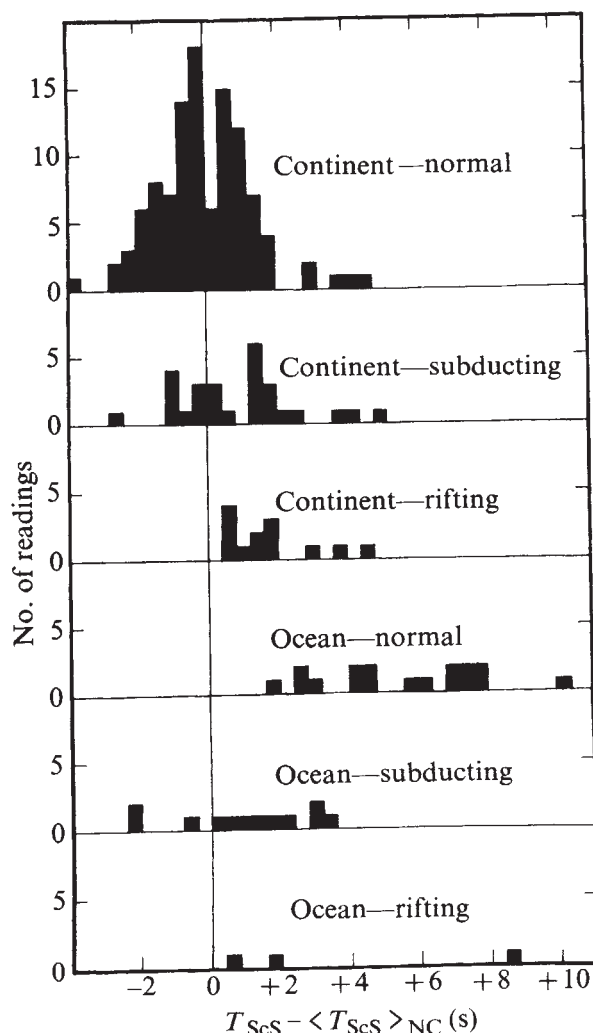
Fig. 1 Differences in one-way vertical shear-wave travel time from a depth of 700 km to the surface predicted by various velocity models. The JB time (143.6 s, ref. 16) has been subtracted to form residuals. Models UTD124A (ref. 14) and B1 (ref. 15) are gross Earth models which satisfy the free oscillation data; model SLUTD1 (ref. 29) is a shear-wave velocity model derived from continental based travel times; and models S1 and O1 (ref. 11) are regionalised models which satisfy the pure-path surface-wave dispersion data for shields and oceans, respectively.

Direct measurements of vertical travel-time differences for shear waves propagating through the continental and oceanic upper mantles have been made using ScS and multiple ScS phases. These times confirm the existence of a 3–5-s difference inferred from the free oscillation work. Figure 2 summarises the ScS travel-time differences from deep focus events observed by Sipkin and Jordan¹⁸. ScS was used because its travel-time curve is not sensitive to radial variations in velocity and because it seems to be less affected by lower mantle heterogeneity than the direct S phase (see below). In this study the seismic stations were crudely classified into six categories, each representing a particular tectonic setting. The four stations placed into the "normal oceanic" category were Kipapa (Oahu), Afiamalu (Samoa), Raratonga and Bermuda. The ScS times from these stations all show large delays relative to the average "normal continental" times; the average residual is close to +5 s.

There is some doubt as to whether or not the mantle beneath these oceanic island stations is truly "normal". After all, islands are by definition anomalous, and many may be the sites of convective upwelling in the mantle^{19,20}. A further test for the existence of large oceanic delays is provided by the observation of time differences between the arrivals of ScS and ScSScS, an S phase which is twice reflected off the core and once reflected off the surface. ScSScS–ScS time differences are insensitive to the velocity structures in the vicinity of the source and receiver, as well as to any mislocation errors. By using geometries where the surface bounce of ScSScS occurs beneath a deep ocean basin, vertical travel-time differences beneath continents and normal ocean can be obtained. An analysis of 64 ScSScS–ScS time differences has yielded one-way vertical travel-time differences between oceans and continents which average +3 to +4 s (S. A. Sipkin and J.H.J., unpublished). Although somewhat less than the average delay from the island stations, these oceanic delays are compatible with the times inferred from free oscillations.

When combined with the regional surface-wave dispersion data, the large vertical travel-time differences between continents and oceans imply that continent–ocean heterogeneity extends to depths exceeding 400 km^{13,18}. The velocity

Fig. 2 A histogram plot of ScS residuals for each of Sipkin and Jordan's¹⁸ tectonic classification types. The residuals have been formed by subtracting the average normal continental residual for each earthquake studied, a procedure which eliminates errors due to hypocentral mislocation.



differences in the upper 400 km of the mantle are reasonably well constrained by the surface-wave data, and these differences can account for only a second or two of the travel time difference. To satisfy the observed oceanic delay of +3 to +5 s requires the existence of deeper velocity differences.

This inference is supported by the free oscillation results. The models derived from the free oscillation data are typically characterised by lower shear velocities in the depth range 400–700 km than models derived from continental body wave data¹³.

A sketch of possible shear velocity profiles for shield and oceanic upper mantles is given in Fig. 3. These velocities are tentative; it is unclear exactly how the velocity differences are distributed with depth. For example, it is not known if the depths of the major discontinuities are displaced. Analysis of internally reflected waves shows some promise of answering questions such as these²¹, but the precise delineation of oceanic upper mantle structure must await long-line refraction studies using seismometers on the ocean bottom.

The comparisons made here contrast the "typical" structures of the continents and the oceans. Clearly, important variations in mantle structure exist within the confines of the continental margins and the ocean basins. In North America at least, the seismic data suggest that some or even most of these regional variations are associated with the transition from shield to oceanic structure; that is, much of the continent-ocean transition occurs in regions overlain by continental crust. On a transit south or south-west from the Canadian shield there is a general increase in P and S station anomalies¹ and the appearance of a well developed low velocity zone². Across the Rocky Mountain Front this transition is quite abrupt, and the entire western United States may be underlain by mantle that is essentially oceanic in character. It is important to chart the shield-ocean transition across a quiescent continental margin, such as the eastern coast of North America, again, an exercise requiring seismometers on the ocean bottom. Across such a margin

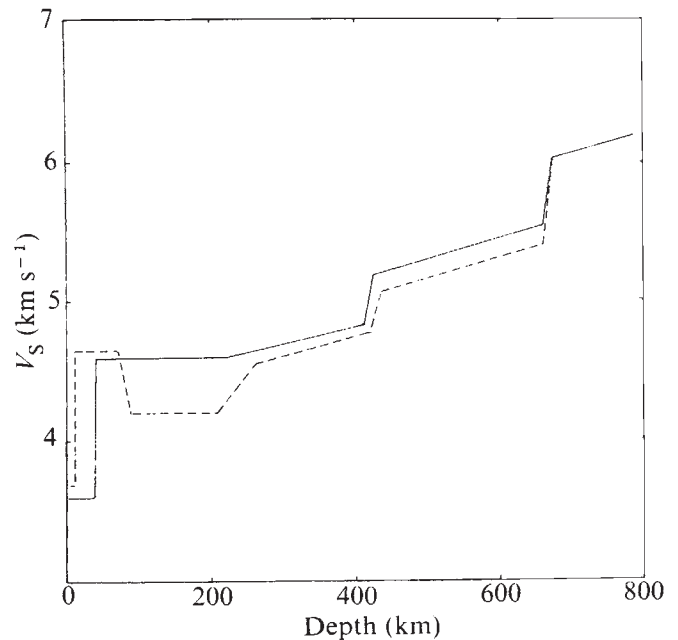


Fig. 3 Possible models of the shear-wave velocity structures beneath continents and oceans. The models are compatible with the surface-wave dispersion data and have a difference in one-way vertical travel time of 3.1 s. —, Shield; ---, ocean.

the lateral velocity gradients may be very large in magnitude.

I have presented elsewhere¹³ a possible model for the upper mantle which can account for the existence of deep-seated continent-ocean velocity contrasts and which is consistent with our knowledge of the upper mantle thermal regime. Briefly stated, this hypothesis postulates that,

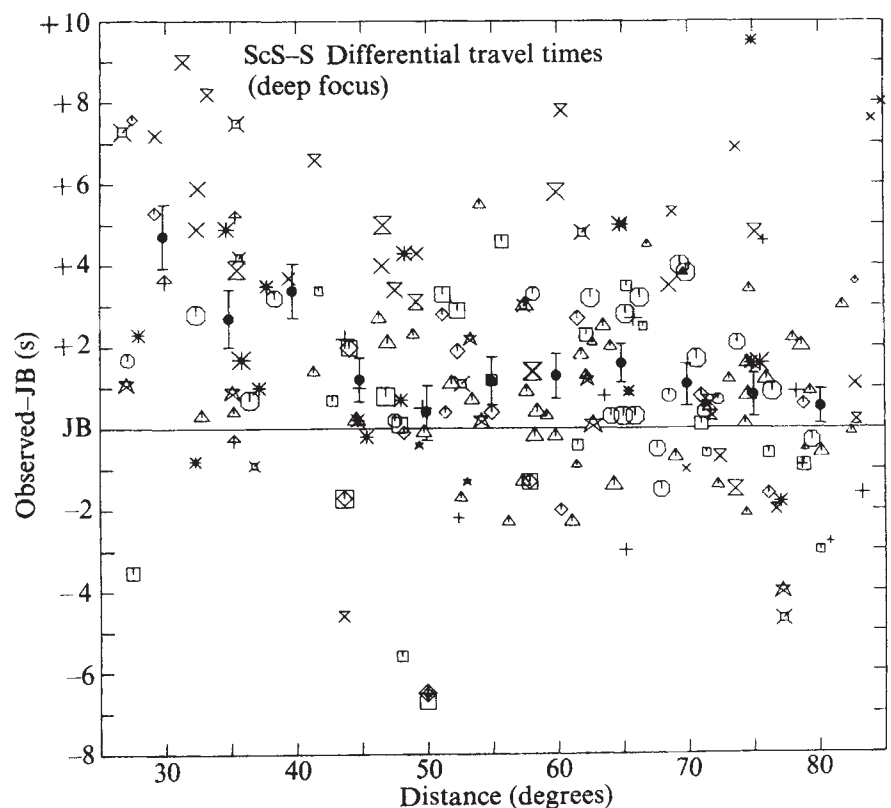


Fig. 4 ScS-S residuals at WWSSN stations from a global distribution of deep-focus events³⁰. Black dots are 5° cell means; error bars represent 1 s.e.m. Each symbol corresponds to a different event, and the size of the symbol is proportional to an assigned subjective quality of the reading.

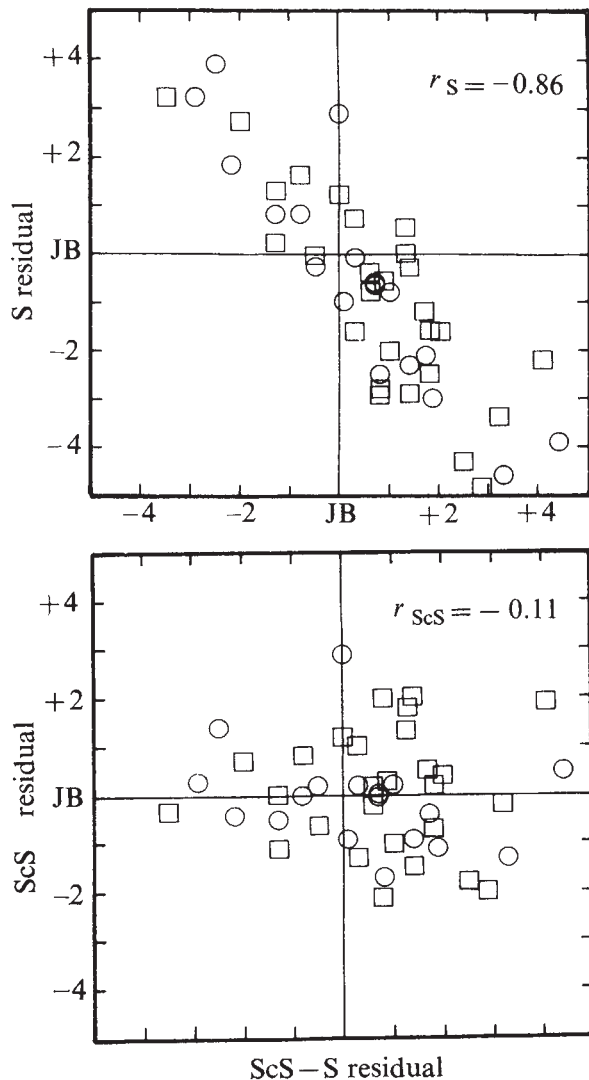


Fig. 5 Correlation plots of S residual versus ScS-S residual (top), and ScS residual versus ScS-S residual (bottom) for times from two Peru-Brazil deep-focus events recorded at United States and Canadian stations²⁸. r_s and r_{scS} are the respective sample correlation coefficients.

beneath the ancient shields, the region which translates coherently in the course of horizontal plate motions (the tectosphere) extends to at least 400 km depth and may occupy the entire upper 700 km of the mantle. Within the continental tectosphere the temperatures are lower, the thermal gradients are super-adiabatic, and the dominant mechanism of heat transport is conduction, not advection. To stabilise the vertical super-adiabatic temperature gradients beneath the shields and the horizontal temperature gradients within the transition from shield to oceanic structure, the model invokes compositional gradients, regions of lower potential temperature consisting of material with intrinsically lower densities.

Admittedly, this model entails a radical departure from the accepted assumption that the thickness of the plates and the lithosphere are identical; it rejects the hypothesis that the continents are decoupled from the mantle at the classical lithosphere-asthenosphere boundary. Furthermore, if the model is correct, continental drift is no longer a simple consequence of plate tectonics. But, in spite of these uncomfortable implications, the model does not seem to be at variance with any significant constraint and thus deserves at least tolerant consideration.

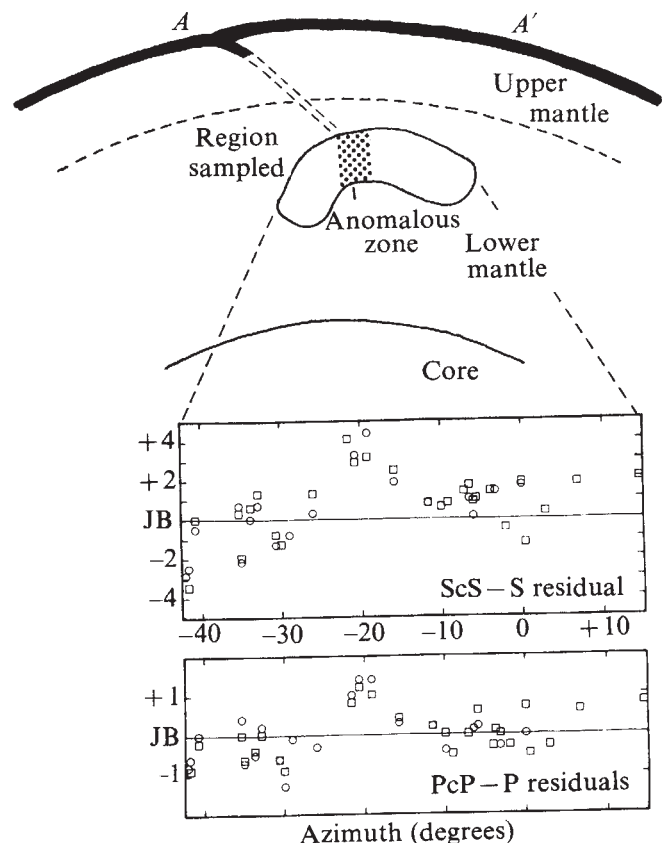
In any case, the seismic data presented here, as well as the old problem of the differences in heat fluxes from the sub-continental and sub-oceanic mantles, require some explanation. The only alternatives I have been able to envisage demand unusual dynamical configurations that seem even more improbable than the tectospheric model.

Lower mantle heterogeneity

Although lateral variability in upper mantle velocities has been known to exist for some time, the existence of lower mantle lateral heterogeneity has been only recently demonstrated. Of course, the difficulty with seeing deep heterogeneity is that it is obscured by geographical variations near the surface, which are generally of larger magnitude in terms of seismic effects. The important work on the subject has been accomplished using a variety of high-resolution techniques, which include body-wave amplitude analysis²², the use of large-aperture seismic arrays²³⁻²⁶, as well as the more conventional measurement of travel times^{27,28}. The most significant result for mantle dynamics to emerge from these studies is the detection of variations in the deep mantle beneath subduction zones.

Data that are particularly suitable for the study of lower mantle structure are the time differences between direct and core reflected phases (PcP-P and ScS-S). At distances greater than 30° or so these differential travel times are not much affected by even gross velocity variations in the crust and uppermost mantle, and they are relatively insensi-

Fig. 6 ScS-S and PcP-P residuals as a function of azimuth from two Peru-Brazil events²⁸. The vertical section through the mantle shows that the large positive residuals in the azimuth window from -15° to -25° define an anomalous zone which lies along the projection of the Benioff zone beneath the Middle America Trench. The section is located on a map view in Fig. 7.



tive to event mislocations as well. Hales and Roberts²⁹ presented 30 observations of ScS–S differential travel times to estimate the radius of the core–mantle boundary. In this study they noticed that the differential travel times showed an unexpectedly large scatter (about 10 s). They speculated that this scatter was due to lateral heterogeneities in the lower mantle, although they did not rule out the possibility of mislocation errors or variations near the source.

I have obtained a considerably larger set of ScS–S times from World-Wide Standard Seismographic Network (WWSSN) recordings of a global distribution of deep-focus events for use in gross Earth modelling^{15,30}. These observations, presented in Fig. 4, show a scatter similar to those obtained by Hales and Roberts. The use of deep-focus events eliminates the near-source traverse through the upper mantle and lends additional support to the hypothesis that the dispersion of times results from velocity heterogeneity in the lower mantle.

A detailed examination of differential travel times, both PcP–P and ScS–S, from two deep focus events in the Peru–Brazil region of South America²⁸ elucidates further the source of the scatter. First, a strong correlation between PcP–P and ScS–S times exists for these events, suggesting the scatter is not simply due to reading errors or some other random noise process. Second, the ScS–S differential times show a strong negative correlation with absolute S times and do not correlate with ScS times (Fig. 5). This implies that the scatter arises from variations along the path of the direct phase, not the core reflection. (This was part of the rationale for using ScS to probe upper mantle heterogeneity, discussed above.) If this interpretation is generally correct for ScS–S, the magnitude of the scatter in these times can be used to estimate the degree of lower mantle heterogeneity. The times in Fig. 4 show a total variation of about 8 s. Supposing the typical scale length for lower mantle heterogeneity to be 1,000 km (an arbitrary number), we obtain velocity contrasts along the path of the S wave of about 5%, a surprisingly high value.

A third result found in the study of the Peru–Brazil data was that the residuals of both PcP–P and ScS–S show a coherent azimuthal dependence (Fig. 6). Of particular note is the grouping of large residuals in the azimuth window -15° to -25° . The rays corresponding to these anomalous times traverse the lower mantle through a region which lies along the extension of the Benioff zone beneath the Middle America Trench, as illustrated in Fig. 6. The existence of an anomaly at approximately this location is supported by two other observations. Davies and Capon³¹ reported anomalous body-wave multipathing at the Large-Aperture Seismic Array (LASA) in Montana for a northern Columbian event, which they suggested might be due to lower mantle heterogeneity. The great-circle ray for this earthquake passes through the anomalous zone defined by the Peru–Brazil data (Fig. 7). In a separate study, Sheppard³² presented an array diagram for the Norwegian Seismic Array (NORSAR), and he noted the existence of a probably source-related anomaly for earthquakes in central America. Events in the Oaxaca district of Mexico separated by as little as 75 km in actual location show array mislocations of as much as 700 km. The magnitude of this splitting must mean that the velocity heterogeneity responsible extends into the lower mantle. Again, the ray paths lie near the anomalous zone delineated by the differential travel-time data (Fig. 7).

Not only do the differential travel times define the location of the anomalous zone, but the positive residuals indicate that the anomaly is characterised by unusually high velocities for both P and S waves. To explain this feature as a thermal anomaly, we found that temperature contrasts of at least 200°C were required, the anomaly being cooler than the surrounding mantle. Thus, the interpretation suggested by these data, and adopted by Jordan

and Lynn is that this high velocity anomaly beneath the Caribbean marks the site of cold material descending in a convecting mantle. The implication of this statement is that the lithospheric slab, or some portion of the slab and its adjacent mantle, penetrates to a depth which exceeds 800 km and which may be greater than 1,400 km.

Evidence is accumulating for deep lateral structure beneath other subduction zones, although the results are less decisive than those for the study described above. Weichert²⁵ has attributed certain $dT/d\Delta$ and azimuth anomalies for P waves observed at the Yellowstone array to lateral velocity gradients near 800 km depth beneath the Aleutian Arc. Davies and Sheppard²⁴ in their analysis of the LASA array diagram noted that significant changes in the mislocation vectors for some western Pacific regions correlated with transitions from one arc to another, suggesting the anomalies were due to a structure near the source structure. Powell (unpublished), using LASA and two other large arrays in the western United States, has found that this correlation also exists for other regions, notably the Tonga–Fiji Arc. She has used ray-tracing

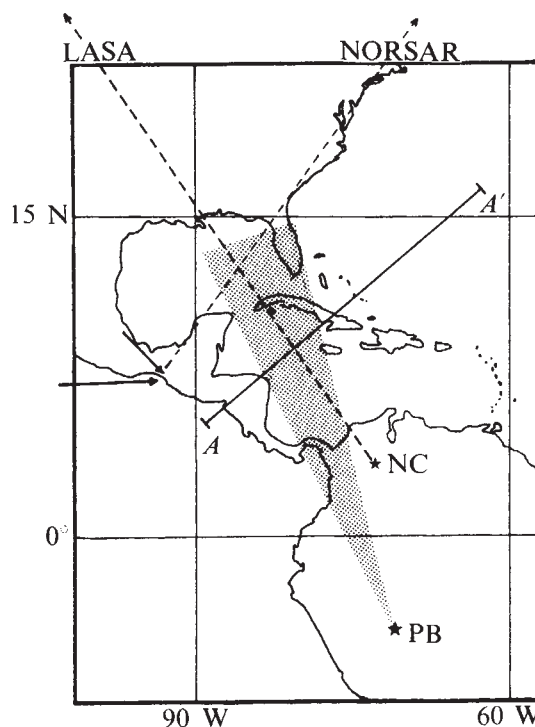


Fig. 7 Map of the Caribbean area. Shaded region is the surface projection of the mantle below 600 km traversed by rays with large positive residuals from two Peru–Brazil (PB) events²⁷. Also shown are the great-circle path between a northern Columbian (NC) event and LASA, for which Davies and Capon³¹ have observed body-wave multipathing, and the great-circle path between two Mexican events and NORSAR, which show large differences in array mislocation. The arrows are the NORSAR mislocation vectors for these earthquakes³².

techniques to investigate the sources of the anomalies and concludes that lateral velocity variations are present at depths exceeding 800 km. Engdahl³³ has observed travel-time differences at Alaskan stations from deep-focus events in the Tonga–Fiji region which are anomalously large and which require that the plate effects extend deeper than 650 km.

The conclusion that descending lithospheric slabs penetrate deep into the mantle cannot be disputed on

theoretical grounds. A numerical study by Schubert, Yuen and Turcotte³⁴ has demonstrated that temperature contrasts of 700 °C can exist between the slab and the adjacent mantle at 800 km depth, which is consistent with the constraints established in the Caribbean study.

Lateral structure in the mid-mantle beneath subduction zones is perhaps sufficient to explain the scatter in ScS-S times shown in Fig. 4. This hypothesis cannot, however, account for all of the heterogeneity in the lower mantle. Julian and Sengupta³⁷ have found a significant increase in P-wave travel-time residuals at epicentral distances beyond 70° which seems to be associated with lateral structure at depths greater than 2,600 km. These variations show no obvious correlation with surface tectonics.

Actually, the lowermost mantle, which includes Bullen's "region D", has been known to be laterally variable for some time. Phinney and Alexander²² reported geographical variations in the amplitude decay of core-diffracted P waves, a measurement quite sensitive to the velocity structure near the core-mantle boundary.

Array studies²⁴ also indicate the presence of heterogeneity in this region, but the problems with eliminating near-source effects are severe. For example, Kanasewich and others³⁵ saw $dT/d\Delta$ and azimuth anomalies at Canadian arrays and at LASA which they ascribed to a feature near the core-mantle interface beneath Hawaii. (These anomalies were also noted by Davies and Sheppard, ref. 24.) The observation provoked much excitement, since it seemed to confirm a deep mantle source for the Hawaiian hotspot, first suggested by Wilson and Morgan¹⁹. But a recent study by Okal and Kuster³⁶, who looked at the deep mantle beneath Hawaii using the French Polynesian array, failed to support this hypothesis, and Powell (unpublished) has shown that the observations reported by Kanasewich and his colleagues probably result from near-source structure of the sort described above.

Nevertheless, the mantle directly above the core-mantle boundary seems to be quite inhomogeneous, probably on many scales. Julian and Sengupta found lateral correlations in residuals with scales on the order of 1,000 km. Much smaller scale heterogeneity in this region (scale lengths on the order of tens of kilometres) has been postulated by Haddon *et al.*³⁷ to account for the unusual characteristics of certain arrivals precursory to the phase PKIKP.

The relationship of this heterogeneity near the core-mantle interface to mantle dynamics remains obscure.

Implications of lateral heterogeneity

Although the formal study of the Earth's lateral heterogeneity is still in its infancy, two conclusions with profound implications for mantle dynamics seem to be required by the available data. (1) Contrasts in the seismic velocity profiles beneath the stable continental shields and the ocean basins persist to depths exceeding 400 km and may extend as deep as 700 km. (2) Strong lateral velocity gradients exist at depths greater than 800 km beneath many subduction zones. Beneath at least one of these, the Middle American Trench, is a zone of anomalously high seismic velocity which extends to perhaps 1,400 km depth.

I interpret these results in terms of the following hypotheses. (1) Beneath the ancient shields the mass which translates coherently in the course of horizontal plate motions (the tectosphere) is at least 400 km thick. Within this tectosphere, the temperatures are lower, the thermal gradients are super-adiabatic, and the dominant mechanism for heat transport is conduction. Anomalous temperature gradients are dynamically stabilised by compositional gradients¹². (2) The lithospheric slab descending beneath subduction zones (and probably some of its adjacent mantle)

penetrates well into the lower mantle before being thermal equilibrated. These, of course, are only hypotheses formulated from limited data, and they must be vigorously tested before being taken too seriously.

Should either prove to be correct, however, one implication may be drawn which constrains the nature of mantle dynamics: more than just the Earth's crust and upper mantle participates in whatever form of convection is responsible for plate motions. Some sort of deep-mantle convection would be necessary to accommodate the mass flow beneath the continents required by the current geometry of seafloor spreading and destruction, given that the tectosphere is very thick beneath continents. If slabs penetrate the lower mantle, then the implication that deep circulation exists is immediate: we have observed it directly.

Deep-mantle convection is not a radical concept on geophysical grounds, but it does pose a geochemical problem. It has been recognised that the oxidation state (as measured by the ferric-ferrous ratio) and nickel content of rocks seen at the Earth's surface are much larger than would be expected if upper mantle material had even been in equilibrium with the metallic core³⁸⁻⁴⁰. If convective cycling involved material transport at the base of the mantle, in the present epoch or those past, then these observations would be very difficult to explain. On these grounds alone, it may be necessary to postulate the existence of a barrier which prevents the exchange of iron and nickel between the upper mantle and the core. On the basis of the seismic data, however, it is unlikely that this barrier is the entire lower mantle.

As indicated in this report, progress in delineating the Earth's lateral heterogeneities is proceeding rapidly. Much work remains to be done, but it is hoped that the study of lateral variations will provide the keys for unlocking the secrets of mantle dynamics.

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articles

X-ray analysis of glucagon and its relationship to receptor binding

Kyoyu Sasaki, Susan Dockerill, Dorota A. Adamiak*, Ian J. Tickle & Tom Blundell†

Biochemistry Laboratory, School of Biological Sciences, Sussex University, Falmer, Brighton BN1 9QG, UK

X-ray analysis of the pancreatic hormone glucagon shows that in crystals the polypeptide adopts a mainly helical conformation, which is stabilised by hydrophobic interactions between molecules related by threefold symmetry. A model is presented in which the glucagon molecule exists in dilute solutions as an equilibrium population of conformers with little retention of structure, and in which the helical conformation is stabilised by hydrophobic interactions either as an oligomer or as a complex with the receptor.

GLUCAGON is a polypeptide hormone of 29 amino acids (ref. 1 and Fig. 1) which is synthesised and stored in the α cells of the islets of Langerhans of the pancreas. The hormone activates glycogenolysis and gluconeogenic pathways resulting in raised blood glucose levels. Rodbell *et al.*² showed that this is mediated by specific binding to a plasma membrane receptor site on the regulatory component of adenylate cyclase of liver and other cells, which gives rise to an increase of intracellular levels of the second messenger cyclic AMP.

An understanding of the nature of the glucagon-receptor interactions depends critically on a proper description of the conformation of the hormone when bound to the receptor. Solution studies show that glucagon exists as an equilibrium population of conformers in dilute aqueous solution^{3,4} but the structure becomes more ordered on self association^{5,6} and in the presence of detergents⁷ or lipid micelles⁸. Preliminary X-ray studies^{9,10} indicated that the crystal structure is mainly helical. We have now carried out a full X-ray analysis at ~ 3.0 Å resolution using the method of isomorphous replacement and anomalous scattering. We describe here our findings on the crystal structure of glucagon; we discuss the relevance of this structure to solution studies, to storage in α cells and to interactions with the receptor; and we compare the structure of this hormone with that of insulin, the only other polypeptide hormone whose structure has been determined by X-ray analysis¹¹.

Glucagon crystals

Glucagon was first crystallised by Staub *et al.*¹² and the space-group (P2₁3) and unit cell parameters ($a = 47.91$ Å) of the cubic crystals were reported by King⁹. We found that large crystals (> 1 mm diameter) could be grown at pH 9.2 in phosphate buffer by warming a 0.3% solution of glucagon to

50 °C and cooling slowly in a Dewar packed in a hot-box. As pH 9.2 is higher than physiological pH (~ 7.4), we studied the stability of the crystals as a function of pH. Transfer of the crystals to a buffer (acetate or phosphate) in the pH range 5.8–7.5 leads to the cracking of the crystals, but not to their complete disintegration. The crystals still diffract X rays; the maximum resolution (~ 3 Å) of the data is not affected but the mosaic spread is increased. The unit cell dimensions are decreased ($a = 47.1$ Å) and the diffraction pattern shows large intensity changes. Crystals identical to these, but uncracked, can be grown from dilute solutions of glucagon at pH 6.0, but the decreased solubility of glucagon at this pH makes it difficult to grow large crystals. These experiments suggest that the molecular structure is flexible in the crystals as well as in solution.

This made us cautious in our attempts to prepare heavy atom derivatives. W. N. Lipscomb (unpublished) noticed changes in intensities which indicated changes in structure at high pH when heavy atom salts were added. We have shown that metal cations such as Ag⁺ and Hg²⁺ and complexes such as PtCl₄²⁻ change the structure at pH 9.2 towards the lower pH crystal form; but many other anionic complexes such as Au(CN)₂⁻ and UO₂F₅³⁻ cause a change in the reverse direction. Such changes undoubtedly account for the problems encountered by previous workers using the method of isomorphous replacement.

Determination of the structure

We decided to attempt the X-ray analysis of crystals soaked in acetate buffer in the pH range 5.6–7.5. This was for three reasons. First, the pH is closer to physiological pH than at pH 9.2. Second, we found the structure less susceptible to small changes of pH than at pH 9.2. Finally, acetate anions do not form insoluble complexes with metal cations such as Ag⁺, UO₂²⁺ and Sm³⁺; on the other hand, these cations have insoluble phosphates and form insoluble hydroxides at pH 9.2.

Soon after our preliminary studies on heavy atom soaking and pH dependence of the crystal structure, W. N. Lipscomb of Harvard University sent us a summary of his X-ray studies in the pH range 9.5–8.5 which we found very helpful. Three heavy atom derivatives using Ag⁺, PtCl₄²⁻ and Pt(NO₂)₄²⁻, respectively, were prepared using the conditions shown in Table 1. The three derivatives gave similar but not identical intensity changes in the diffraction patterns. Ag⁺ gave a highly isomorphous derivative but the platinum derivatives gave rise to small changes of cell dimensions and the data were attenuated at interplanar spacings of $d < 3.5$ Å.

Three dimensional X-ray data were collected on a Hilger and Watts four-circle diffractometer using a new method for calculation of integrated peak intensities of reflections¹³. For the native and PtCl₄²⁻ glucagon crystals three equivalents

*Present address: Uniwersytet im Adama Mickiewicza, Poznan, ul. Grunwaldzka 6, Poland.

†To whom reprint requests should be addressed.

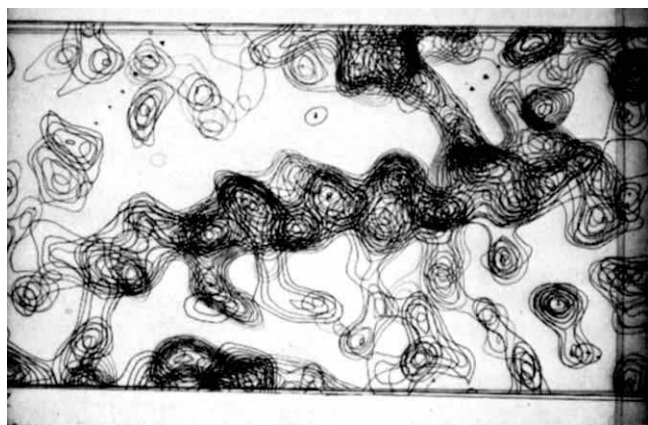
of arginine and lysine have weaker density. In an α -helical conformation, residues 10–25 have a very approximate twofold symmetry as indicated in Fig. 1 and so the helix fits tolerably, but less well if the direction of the polypeptide is reversed. The correct chain direction was confirmed by the better fit of tryptophan 25, by the well defined electron density of phenylalanine 6 and the continuous density corresponding to residues 1–5 in a non-helical conformation.

Two observations suggest that the polypeptide chain of residues 1–5 is flexible: the density of residues 1–5 is weaker than that of the helix and preliminary studies on the pH 9.2 crystal form indicate changes in the conformation of these residues. Change of pH from 9.2 to around 6 will almost certainly lead to protonation of the amino terminus, and probably formation of an ion pair with the carboxy terminus of the glucagon molecule in the next unit cell. This may be the explanation for the rearrangement indicated in the crystals at pH 9.2. The metal ions Ag^+ , PtCl_4^{2-} and $\text{Pt}(\text{NO}_2)_4^{2-}$ bind between methionine 27 and the N terminus, and their substitution at this position probably induces changes in conformation and crystal packing, similar to those induced by lowering the pH.

The conformations of the non-polar residues in the regions 6–14 and 22–27 are shown in Fig. 3. In the crystal structure they are packed against equivalent residues of other molecules to give hydrophobic regions. The threefold axes of the cubic cell pass close to the glucagon molecule (Fig. 2), and contacts arise between molecules related by these symmetry operations. Figures 4 and 5 show trimeric arrangements of glucagon viewed down the two threefold axes which give rise to extensive intermolecular contacts.

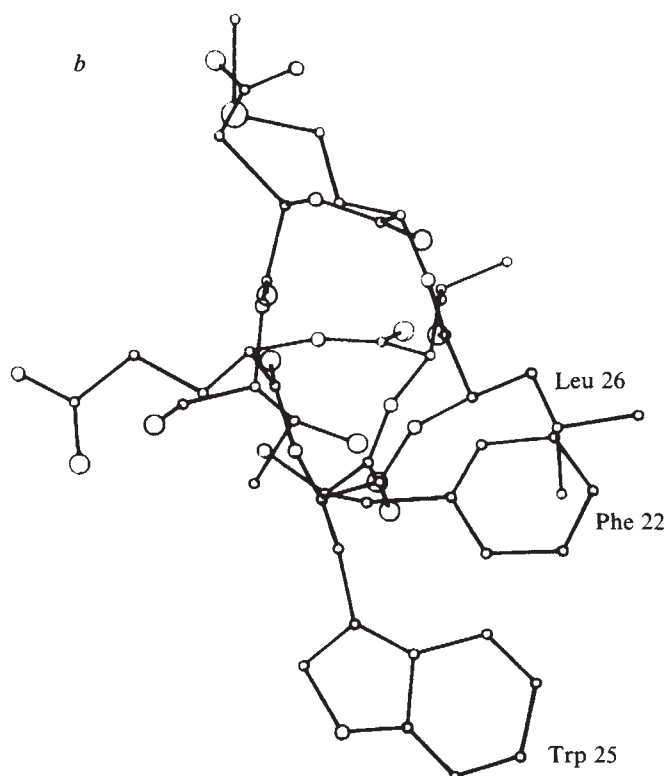
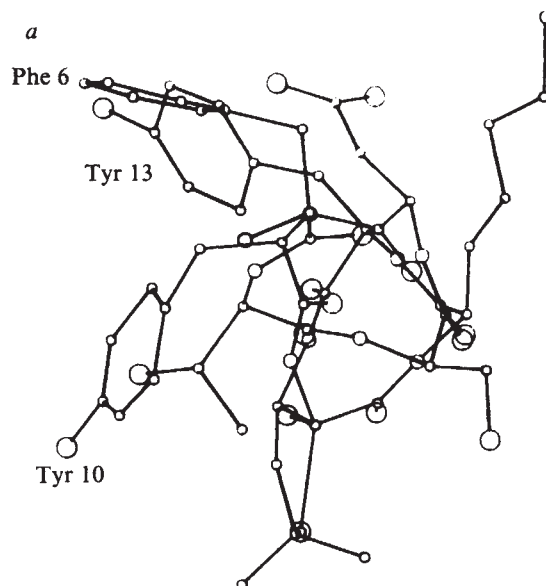
Figure 4 shows the association of the glucagon molecules through contacts between the sidechains of tryptophan 25, leucine 26 and phenylalanine 22 of one molecule and tyrosine 10, tyrosine 13 and phenylalanine 6 of the second. This leads to a triangular structure with the central region around the threefold axis containing solvent and the sidechains of arginines and aspartic acids. In Fig. 5a the association around a second threefold axis leads to contacts between equivalent amino acids at the carboxy terminus of the molecule including phenylalanine 22, valine 23 and leucine 26, which are shown enlarged in Fig. 5b. This hydrophobic region is extended by close packing of sidechains of phenylalanine 6 and tyrosine 10 of other molecules so that the region of non-polar contacts described first is continuous with the second. The result of this complicated cubic close packing is to bury the hydrophobic regions shown in Fig. 3, which are formed when the molecule adopts a helical conformation.

Fig. 2 Part of the $\sim 3 \text{ \AA}$ electron density map viewed along a cell axis. Triangles indicate the positions of the threefold axes in the unit cell.



As King⁹ suggested, the crystal structure approximates to a cubic packing of cylinders; the individual protomers do not have a globular structure which characterises proteins studied by X-ray analysis.

Fig. 3 The conformation of residues (a) 6–14 and (b) 22–29 viewed approximately along the helix axis showing the clustering together of hydrophobic residues.



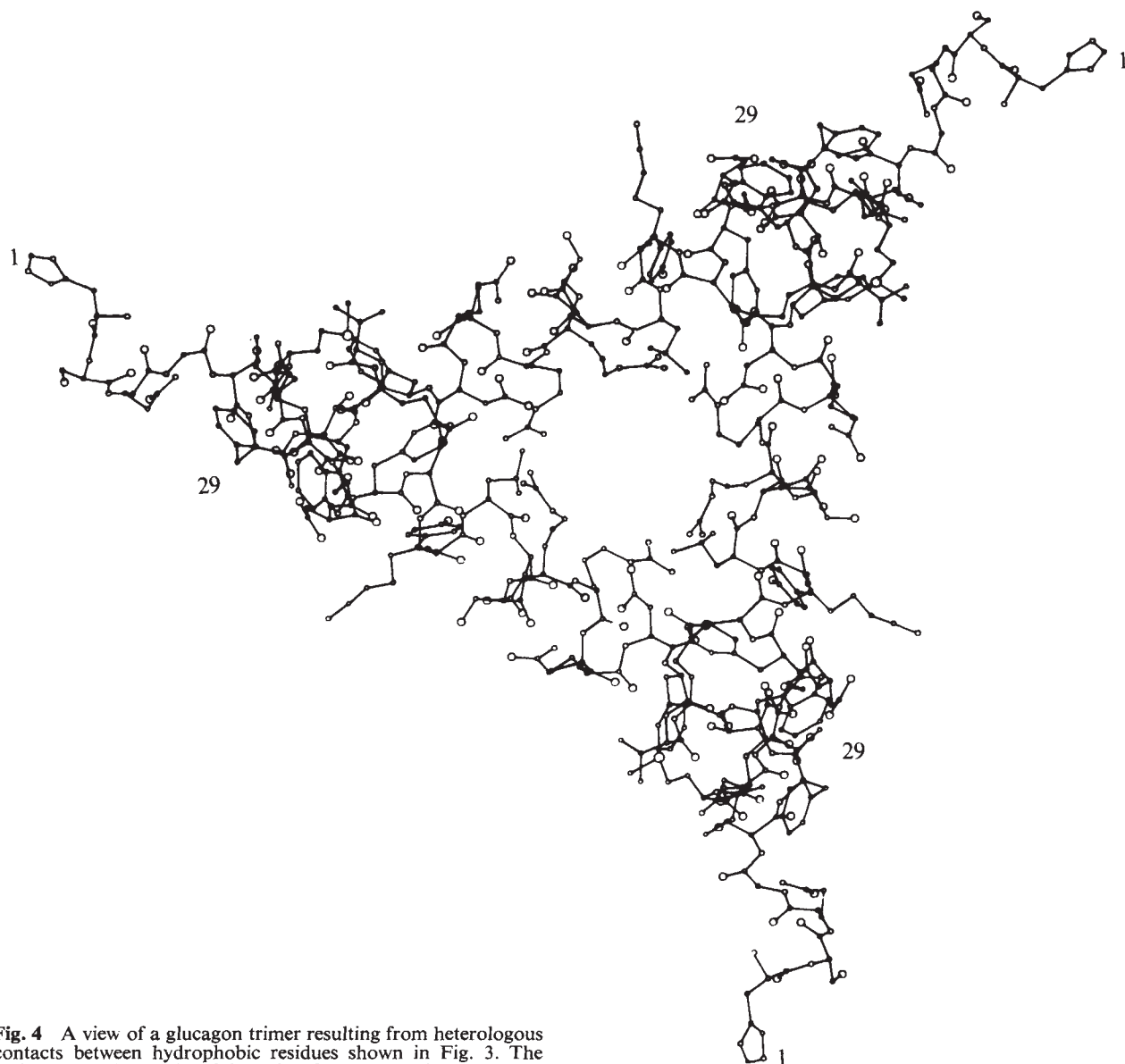


Fig. 4 A view of a glucagon trimer resulting from heterologous contacts between hydrophobic residues shown in Fig. 3. The region around the threefold axis of symmetry in the centre comprises hydrophilic residues including arginines and aspartates.

Solution structure

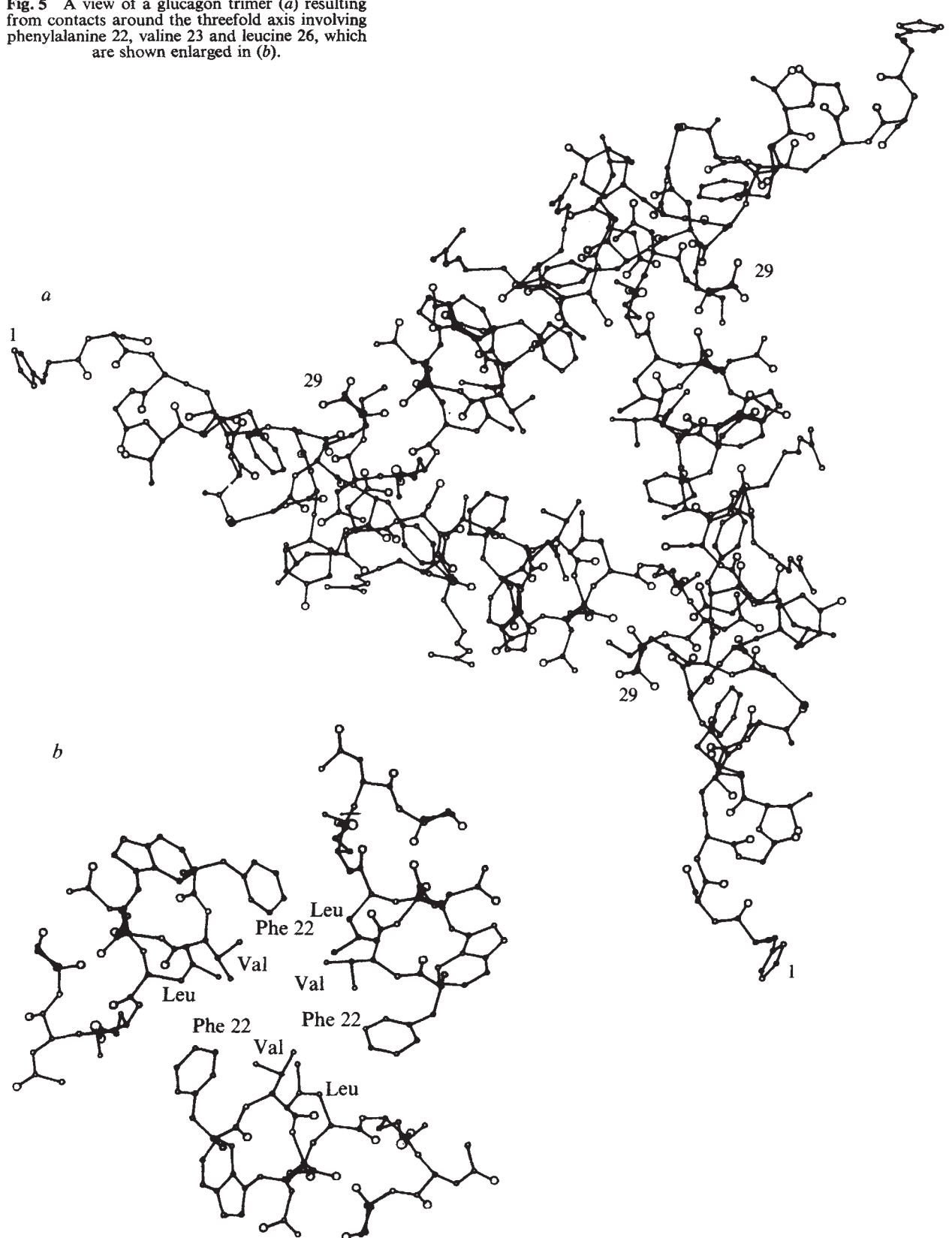
In solution at high dilutions glucagon does not have a highly ordered conformation^{2,3}; it is probably flexible, existing as an equilibrium population of conformers. As a result of the low solubility at neutral *pH*, most solution studies have been carried out at *pH* 10. At this *pH*, optical rotatory dispersion⁴ and circular dichroism⁵ indicate that a largely α -helical conformation is induced by concentrating to 3 mg ml^{-1} , and that the conformational change accompanies an association of molecules probably to trimers^{4,5} and possibly to higher oligomers²¹. The association is accompanied by changes in ellipticity and wavelength in the circular dichroism arising from the tyrosine and tryptophan chromophores⁵. These observations can be explained reasonably well in terms of the crystal structure. The association of monomers to trimers resulting in an α -helical conformation probably corresponds to the formation of the trimer shown in Fig. 4, as this arrangement requires a helical conformation, and unlike the trimer shown in Fig. 5, it would give rise to changes in the near ultraviolet circular dichroism due to both tryptophan and tyrosine. It is also consistent with the observation²² that association affects the *pK* of the phenolic hydroxyl of tyrosine 10, which in the crystal trimer appears to be more buried than tyrosine 13.

The far ultraviolet circular dichroism of the glucagon molecules at *pH* 10 and 3 mg ml^{-1} is very similar to the spectrum of zinc insulin hexamers which has about 50% of its residues in right-handed helical conformations¹¹. This percentage is less than that observed in the crystal structure of glucagon. The difference, however, may not be real; it may be a consequence of the rather irregular helix in glucagon. Alternatively it may arise partly from differences in conformation between *pH* 10 and *pH* 5.8–7.5 where the crystal structure is stable, and partly from a tightening up of the helix as the trimers are associated to higher oligomers as seen in the crystals. Whatever the explanation, the crystal structure demonstrates why a helical arrangement is favoured by association of glucagon molecules; it leads to burying of hydrophobic residues.

Receptor binding

Rodbell *et al.*²³ have found evidence that discrete regions of the glucagon molecule are important to receptor binding and activity. On the basis of studies with des-histidine-glucagon and smaller fragments of the sequence, they suggest that while essentially the entire molecule of glucagon must be considered to be the active species, the histidine at the amino terminus is relatively unimportant to receptor binding.

Fig. 5 A view of a glucagon trimer (*a*) resulting from contacts around the threefold axis involving phenylalanine 22, valine 23 and leucine 26, which are shown enlarged in (*b*).



At physiological concentrations ($\sim 10^{-11}$ M) glucagon must exist as a monomer and so it is unlikely that glucagon trimers or higher oligomers have any role in receptor binding. But the decreased stability with reduced temperature or with the addition of urea indicates that the glucagon receptor complex is stabilised by hydrophobic interactions²⁴. We are

therefore not concerned with conformation of the monomer in aqueous solution, but rather with the structure stabilised by interaction with a hydrophobic surface. A helical structure is stabilised by lysolecithin⁸, cetyltrimethyl ammonium bromide⁷ and glycols²⁷ which increase the hydrophobicity of the environment of the glucagon molecule. The crystal structure shows that

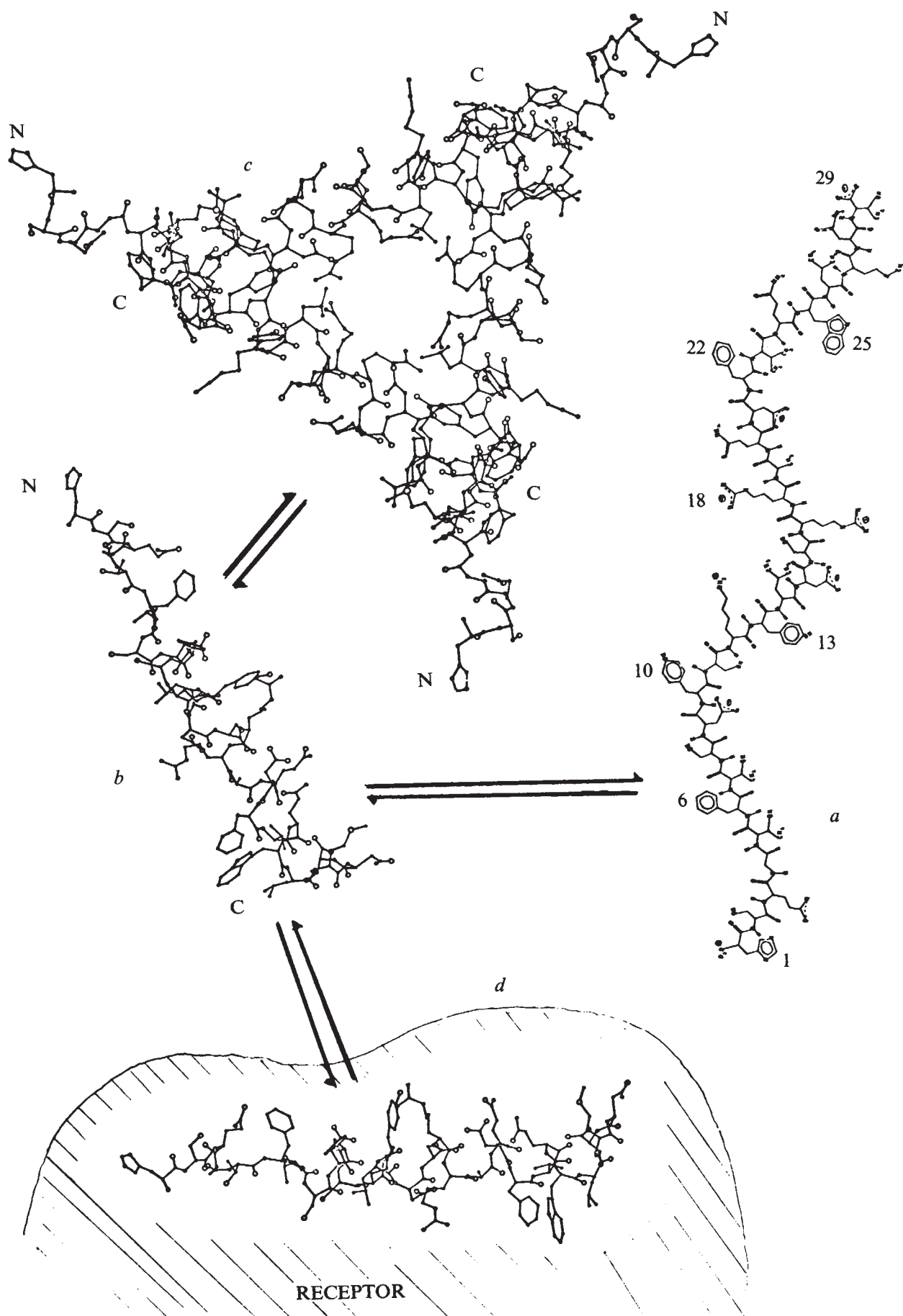


Fig. 6 A schematic representation of the equilibrium between conformers of glucagon. (a) Hypothetical random coil structure in equilibrium with a helical form (b). The helical form is stabilised either as trimers (c) or by association with a receptor (d) by hydrophobic interactions.

a helical conformation is stabilised by association of glucagon molecules to form trimers and higher oligomers. This structure is favoured by hydrophobic contacts because in a helical arrangement non-polar residues are clustered together in two surface regions (Fig. 3). The receptor-bound glucagon may have a similar conformation stabilised by interaction of the glucagon with two hydrophobic regions on the receptor. The importance of both these regions for binding is consistent with the retention of these hydrophobic residues in all active glucagons, the strong receptor binding of des-histidine glucagon, and the inability of either amino-terminal fragments (glucagon 1-21, glucagon 1-23) or carboxy-terminal fragments (glucagon 20-29, glucagon 22-29) to compete with labelled glucagon at its receptor²⁴. The hydrophilic residues adjacent to the two hydrophobic regions may give rise to further stabilising interactions in the glucagon-receptor complex and this may explain the conservation of most of these residues in glucagons from different species. Carbamylation of lysine 12 has little effect on biological activity *in vitro*²⁵; however, modification with a larger group such as trinitrobenzyl²⁶ decreases the biological activity. It also increases the helical content; it probably interacts with the adjacent tyrosine 10 and tyrosine 13, so stabilising the helix but interfering with receptor binding.

This model implies that the glucagon molecule exists in solution as an equilibrium population of conformers with little retention of structure, and that the helical conformation is stabilised by hydrophobic interactions either as an oligomer or as a complex with the receptor, as shown in Fig. 6. This scheme is reminiscent of that of Schwyzer for flexible hormones²⁸. An alternative model would involve an induced fit for the interaction of the glucagon molecule with the receptor²⁹. For instance the C-terminal residues 22-27 containing one hydrophobic area may bind initially in a helical arrangement and induce a helix in the residues 6-21 in a stepwise fashion so that the second hydrophobic area can also bind.

The length of the largely helical glucagon molecule is over 40 Å and the centres of the two hydrophobic regions are about 20 Å apart. This is a very long structure to be bound to one regulatory unit of adenylate cyclase, which may have a molecular weight of about 26,000 (ref. 30) and it is conceivable that glucagon binds two regulatory subunits, each through one hydrophobic region. If these two units are closely homologous and arranged as a dimer with a twofold axis, this would explain the approximate symmetry of the hydrophobic patches around the centre of the helical region shown in Fig. 1. The hydrophobic groups in the centre may form further stabilising interactions with the receptor in the region between the regulatory subunits. A similar twofold symmetry has been proposed for the active conformation of luteinising hormone releasing hormone³⁰, and it has been suggested that this hormone binds two symmetry related subunits in a similar way to the binding of the allosteric effector 2,3-diphosphoglycerate to haemoglobin.

In the crystal structure the region 1-4 does not form a well defined helical arrangement, but changes its conformations easily. This may be facilitated by the presence of glycine 4; glycine can easily attain conformations which are energetically unfavourable to amino acids with sidechains. It is possible that the flexibility also has a role in biological activity. Once the helical region is bound to the receptor the residues 1-4 may change in conformation to interact with the receptor in a way which gives little further thermodynamic stability to the glucagon-receptor complex but which results in the initiation of the biological response. This would be consistent with the antagonistic action of des-histidine-glucagon.

decreases, rather than increases, glucose levels in the blood. Glucagon is a flexible molecule which easily attains a helical structure in a hydrophobic environment. On the other hand, insulin has a well defined and relatively inflexible globular structure. In the latter case the importance of the three dimensional structure appears to result from the involvement of residues widely separated in the primary sequence in a relatively small receptor binding region¹¹. A stable tertiary structure, however, may have certain disadvantages; it will be degraded more slowly, and, therefore there may be less sophistication in the control of metabolism, as the effect of the hormone might linger on for some time. For insulin the degradation may be facilitated by the presence of a very exposed disulphide at A7-B7. The more flexible structure of glucagon would be more easily degraded by a proteolytic enzyme.

The two hormones, insulin and glucagon, do have similarities. Both associate in concentrated solutions through mainly hydrophobic interactions. The oligomers crystallise *in vitro* and a similar crystallisation seems to be involved in granulation of the hormones in storage. For instance, glucagon granules of certain species are crystalline rhombic dodecahedra (R. Lange and C. Klein, unpublished) and are identical in appearance to the crystals studied by X-ray analysis. Insulin granules are usually crystalline and often have a cubic or rhombohedral close packing of zinc insulin hexamers¹¹. Association of monomers in storage would undoubtedly decrease the rate of enzymatic proteolysis and increase the thermodynamic stability, in addition to providing a concentrated storage form¹¹. In both hormones, the hydrophobic residues which are involved in association also seem to be involved in receptor binding.

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Insulin and glucagon

Our findings on the structure of glucagon contrast strongly with those on the structure of insulin, which is also a polypeptide hormone synthesised in the islets of Langerhans, but which

Tertiary structural differences between microbial serine proteases and pancreatic serine enzymes

Louis T. J. Delbaere, Wendy L. B. Hutcheon, Michael N. G. James† & William E. Thiessen*

Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Although primary structural homology between bacterial serine proteases and those from the mammalian pancreas is slight, two-thirds of the residues in the bacterial enzyme SGPB as seen at 2.8-Å resolution, adopt a similar polypeptide chain conformation to that of the chymotrypsin family. The three major regions of difference show how this family of proteolytic enzymes has developed from the more primitive bacterial to the relatively sophisticated pancreatic enzymes.

THE primary¹⁻⁸ and tertiary⁹⁻¹¹ structural homologies among the three pancreatic serine proteases have provided an excellent case for the common ancestral origin of these mammalian enzymes. There are also examples of serine proteases, from microbial sources, which have some regions of sequence identical to those of the pancreatic enzymes. The α -lytic protease (ref. 12 and refs. therein) from *Myxobacter* 495 and the two enzymes SGPA (ref. 13) and SGPB (ref. 14) from the extracellular culture filtrate (Pronase) of *Streptomyces griseus* are examples of enzymes with the active site sequences Gly-Asp-Ser-Gly-Gly and His-Cys identical to the corresponding sequences in the mammalian enzymes. These enzymes also have an aspartic acid at a position corresponding to the catalytically important Asp-102 of α -chymotrypsin⁹, elastase¹⁰ and trypsin¹¹. The overall sequence homology between the three enzymes from microbial sources and the pancreatic enzymes is, however, so low (< 20% identity, see Table 1) that tertiary structural homology can validly be questioned.

The question of a common ancestral origin of the bacterial and mammalian enzymes cannot be resolved without a determination of the tertiary structure of one of the microbial proteases. To support an argument for the divergent evolution of the Asp-Ser-Gly proteases, McLachlan and Shotton¹⁵ attempted to predict the tertiary structure of α -lytic protease from a knowledge of the polypeptide backbone conformations of α -chymotrypsin and elastase. Even though large deletions and insertions were required to align the two sequences, these authors were able to fit the amino acid sequence of α -lytic protease into the main-chain backbone of elastase with relatively few contraventions of conventional wisdom concerning globular protein conformation. The results of that study gave promise that the tertiary structures of homologous and functionally similar protein molecules could be predicted from a knowledge of the tertiary structure of one member.

Subtilisin BPN' has an active site geometry very similar to that of the pancreatic serine protease¹⁶. There is, however, no primary or tertiary structural homology of this bacterial enzyme with the mammalian enzymes. Thus for these two

protease classes this particular active site has been achieved through convergent lines of evolution.

To establish the relationship of the bacterial serine proteases with those from the mammalian pancreas we have determined the structure of SGPB at 2.8-Å resolution by X-ray crystallography. Some of the conclusions reached by McLachlan and Shotton¹⁵ are borne out by the present analysis; however, three important regions of the microbial enzymes have no tertiary-structural counterpart in the mammalian pancreatic enzymes.

Experimental procedure

Suitable single crystals of SGPB are obtained from 0.7–1.2 M KH_2PO_4 solutions at pH 4.2. These crystals have the symmetry of space group $P2_12_12$ and unit cell dimensions of $a = 44.16$, $b = 108.91$ and $c = 37.34$ Å. Details of the crystal growth, data collection and processing were reported in a preliminary publication on the 4.5-Å resolution structure of SGPB (ref. 17). In the study reported here Ni-filtered $\text{CuK}\alpha$ radiation from a tube operated at 40 kV and 16 MA was used. This reduction in incident beam intensity from conditions reported earlier¹⁷ increased the lifetime of the crystals by a factor of 3.5 and allowed the measurement of the complete 2.8-Å sphere of data (hkl and Friedel mate $h\bar{k}l$, 9,500 reflections) from a single crystal of the native and each isomorphous derivative. The maximum decrease in intensity of the standard reflections was about 12% during the 120 h required to collect these data.

The heavy atom derivatives used in our analysis were prepared by soaking the native enzyme crystals in 1.3 M KH_2PO_4 solutions containing the following heavy atom compounds: 5 mM $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ for 72 h; 2 mM mersalyl for 48 h; a double derivative from 5 mM $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ for 12 h first followed by a mixed solution of 5 mM $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ and 2 mM mersalyl for 24 h; 10 mM K_3IrCl_6 for 72 h and 10 mM K_3IrCl_6 for 24 h. The heavy atom binding sites for these five useful derivatives were identified from cross-phased Fourier maps using the preliminary 4.5-Å phases¹⁷.

A summary of the final heavy atom parameters is included in Table 2. The agreement indices for these five derivatives varied from $R_c = 0.494$ to $R_c = 0.575$, where R_c is the Cullis R factor defined by: $\sum ||F_{\text{PH}} - F_P| - |f_H|| / \sum |F_{\text{PH}} - F_P|$ summed over the centric data only. The variation of the ratio of heavy atom scattering (f_H) to the lack of closure error (E_H) as a function of $\sin^2\theta/\lambda^2$ and the mean figures of merit for these several ranges are drawn in Fig. 1. All these criteria are well within accepted

Table 1 Amino acid identity matrix for the five proteins in Fig. 2

	SGPA	SGPB	α -Lytic	Chymo A	Elastase
SGPA	—	59.0%	35.3%	17.9%	17.9%
SGPB		—	36.0%	17.2%	20.4%
α -Lytic			—	17.7%	19.2%
Chymo A				—	38.8%
Elastase					—

*Permanent address: Chemistry Division, Oak Ridge National Laboratories, Oak Ridge, Tennessee 37830.

†On leave, Department of Structural Chemistry, Weizmann Institute of Science, Rehovot, Israel.

limits for a good determination of the protein phases by the multiple isomorphous replacement method.

Sites 4 and 2 (Table 2) for the $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ derivative were interpreted from a double difference map as a movement of the sulphur side chain of Met-162 on binding the platinum. Site 2 of the 24 h soaked K_3IrCl_6 derivative was interpreted as the loss of one of the bound phosphate groups in the region of the active site Ser-195 (see below). All the heavy atom sites in Table 2 behaved satisfactorily during the least squares refinement cycles.

The electron density map was computed with the native structure factor amplitudes and the best phases¹⁸ derived from the final phasing cycle. Interpretation of this map led to the structure on which the following discussion is based. The phasing program was a locally adapted version of one written by Dr M. G. Rossman; other programs were derived and adapted from the X-ray 70 system compiled by Dr J. Stewart.

Description of structure

To facilitate the comparison of the tertiary structures of the microbial serine proteases with those of the pancreatic enzymes, we have included in Fig. 2 the sequences of SGPB (ref. 14), SGPA (ref. 13), α -lytic protease¹², bovine chymotrypsinogen A (ref. 19) and porcine elastase⁸. The numbering scheme is that of bovine chymotrypsinogen A and chemically similar residues are enclosed in solid lines. The alignment presented here differs in some respects from the alignments previously published¹³⁻¹⁵ and has been based on tertiary structural similarities, a procedure strongly urged by Dickerson²⁰. Figure 2 and Table 2 show that there is considerably more sequence homology among the three microbial serine proteases than between the microbial and pancreatic protease groups.

The amino acid sequence data on SGPB (ref. 14) was invaluable for the interpretation of the 2.8-Å electron density map. The published tentative sequence had to be altered to accommodate two extra residues at positions where the sequence data were incomplete. At residue 65A the sequence Thr-Trp-Ala was altered to Thr-Trp-Trp-Ala to account for an extra lobe of density in the internal hydrophobic core. Chemical and spectrophotometric determinations of Trp in SGPB are also consistent with two tryptophan residues. (L. Jurásek and G. Willick, personal communication). The second region was at residue 186L (Asp-Val-Tyr) which was more consistent with a sequence Asp-Val-Val-Tyr for the interpretation of the

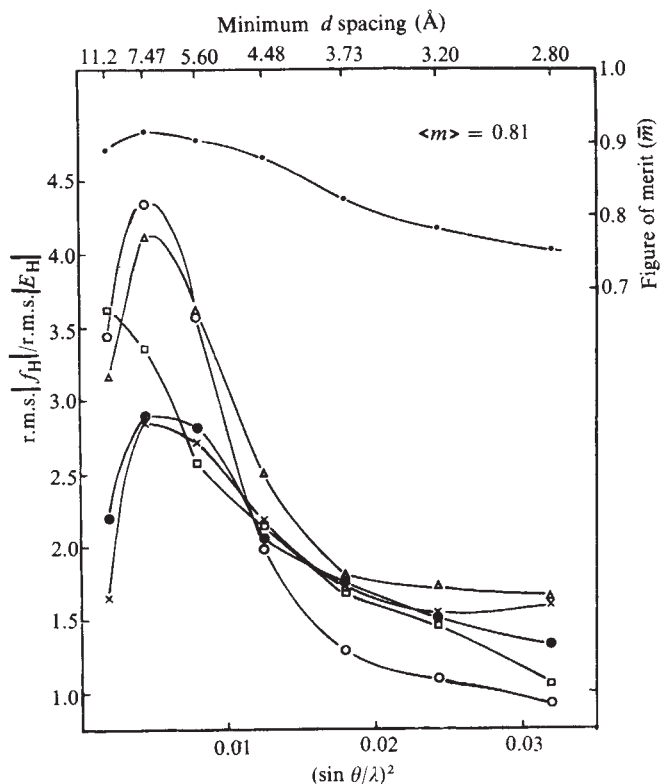


Fig. 1 Variation of the ratio of r.m.s. $|f_H|$ to r.m.s. $|E_H|$ and the figure of merit ($\bar{m}$) as functions of $\sin^2\theta/\lambda^2$. The r.m.s. $|f_H|$ is the root mean square heavy atom structure factor amplitude, r.m.s. $|E_H|$ is the root mean square lack of closure of the phase triangles. The derivatives are represented by the following symbols: $\circ$, $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$; $\times$, mersalyl; $\triangle$, double derivative; $\bullet$, 3-d soaked K_3IrCl_6 ; $\square$, 1-d soaked K_3IrCl_6 .

electron density. The extra valine is consistent with the amino acid analyses of peptides derived from this unsequenced region because of the resistance of a valylvaline peptide bond to acid hydrolysis. There are thus 186 amino acid residues in the SGPB molecule.

Several sections of the 2.8-Å resolution electron density map computed over the region of the active site are shown in Fig. 3.

Table 2 Refined heavy atom parameters. 2.8-Å data

Derivative	Site	x/a	y/b	z/c	A^*	$B^\dagger$
$\text{Pt}(\text{NH}_3)_2\text{Cl}_2$	1	0.8198	0.3490	0.7848	92.7	78.4
	2	0.835	0.3345	0.683	12.3	40.9
	3	0.821	0.3648	0.723	28.0	56.4
	4	0.804	0.3515	0.835	-17.6	5.6
Mersalyl $\ddagger$	1	0.6823	0.4886	0.0317	18.8	0.6
	2	0.3422	0.3624	0.2312	29.7	48.6
	3	0.469	0.408	0.447	4.6	30.1
	4	0.8205	0.3500	0.7841	61.0	70.3
$\text{Pt}(\text{NH}_3)_2\text{Cl}_2 +$ mersalyl	2	0.839	0.337	0.683	4.2	7.2
	3	0.821	0.366	0.715	19.8	94.3
	4	0.807	0.351	0.829	17.0	23.2
	5	0.6820	0.4884	0.0305	24.1	5.0
	6	0.3432	0.3628	0.2286	26.7	25.9
	7	0.468	0.409	0.436	10.3	67.5
	8	0.825	0.321	0.719	7.9	66.3
	9	0.896	0.356	0.747	6.0	35.1
	10	0.293	0.326	0.273	2.6	1.0
	11	0.5	0.5	0.8085	16.4	17.6
K_3IrCl_6 (3-d soak)	1	0.5	0.5	0.8065	25.7	24.5
K_3IrCl_6 (1-d soak)	2	0.4658	0.3903	0.2342	-3.7	59.4

* A is the site occupancy on a relative scale. A negative value of A corresponds to a site occupied in the native protein but not in the derivative.

$\dagger B$ is the isotropic temperature factor coefficient, $\text{\AA}^2$.

$\ddagger$ Mersalyl, o -($\text{NaO}_2\text{CCH}_2\text{O}$) C_6H_4 ($\text{CONHCH}_2\text{CH}(\text{OCH}_3)\text{CH}_2\text{HgOH}$).

	15A	15B	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	36A	36B	36C	37	38	39		
SGPB			Ile	Ser	Gly	Gly										Asp	Ala	Ile	Tyr	Ser	Ser								Thr		
SGPA			Ile	Ala	Gly	Gly											Glu	Ala	Ile	Thr	Thr	Gly							Gly		
α-LP	Ala	Asn	Ile	Val	Gly	Gly											Ile	Glu	Tyr	Ser	Ile	Asn	Asn						Ala		
Chymo A			Ile	Val	Asn	Gly	Glu	Glu	Ala	Val	Pro	Gly	Ser	Trp	Pro	Trp	Gln	Val	Ser	Leu	Gln	Asp	Lys					Thr	Gly	Phe	
Elastase			Val	Val	Gly	Gly	Thr	Glu	Ala	Gln	Arg	Asn	Ser	Trp	Pro	Ser	Gln	Ile	Ser	Leu	Gln	Tyr	Arg	Ser	Gly	Ser	Ser	Trp	Ala		
	40	41	42	43	44	45	46	47	48	48A	48B	48C	48D	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64		
SGPB	Gly	Arg	Cys	Ser	Leu	Gly	Phe	Asn	Val	Arg	Ser	Gly	Ser	Thr	Tyr	Tyr	Phe	Leu	Thr	Ala	Gly	His	Cys	Thr	Asp		Gly	Ala	Thr		
SGPA	Ser	Arg	Cys	Ser	Leu	Gly	Phe	Asn	Val	Ser	Val	Asn	Gly	Val	Ala	His	Ala	Leu	Thr	Ala	Gly	His	Cys	Thr	Ser		Asn	Ile	Ser		
α-LP	Ser	Leu	Cys	Ser	Val	Gly	Phe	Ser	Val	Thr	Arg	Gly	Ala	Thr	Lys	Gly	Phe	Val	Thr	Ala	Gly	His	Cys	Gly	Thr	Val	Asn	Ala	Thr		
Chymo A	His	Phe	Cys	Gly	Gly	Ser	Leu	Ile	Asn					Glu	Asn	Trp	Val	Val	Thr	Ala	Ala	His	Cys	Gly	Val	Thr	Ser	Asp			
Elastase	His	Thr	Cys	Gly	Gly	Thr	Leu	Ile	Arg					Gln	Asn	Trp	Val	Met	Thr	Ala	Ala	His	Cys	Val	Asp	Arg	Glu	Leu	Thr		
	65	65A	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92		
SGPB	Gly	Thr	Trp	Trp	Ala	Asn	Ser	Ala								Arg	Thr	Thr	Val	Leu	Gly	Thr	Thr	Ser				Gly	Ser		
SGPA	Ala	Ser	Trp																			Ser	Ile	Gly	Thr	Arg	Thr	Gly	Thr		
α-LP	Ala																					Arg	Ile	Gly	Gly	Ala	Val	Val	Gly		
Chymo A	Val		Val	Val	Ala	Gly	Glu	Phe	Asp	Gln	Gly	Ser	Ser	Ser	Glu	Lys	Ile	Gln	Lys	Leu	Lys	Ile	Ala	Lys	Val	Phe	Lys	Asn	Ser		
Elastase	Phe	Arg	Val	Val	Val	Gly	Glu	His	Asn	Leu	Asn	Gln	Asn	Asn	Gly	Thr	Glu	Gln	Tyr	Val	Gly	Val	Gln	Lys	Ile	Val	Val	His	Pro		
	93	94	95	96	97	98	99	99A	99B	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119		
SGPB	Ser	Phe						Pro		Asn	Asn	Asp	Tyr	Gly	Ile	Val	Arg	Tyr	Thr	Asn	Thr	Thr									
SGPA	Ser	Phe						Pro		Asn	Asn	Asp	Tyr	Gly	Ile	Ile	Arg	His	Ser	Asn	Pro	Ala									
α-LP	Thr	Phe	Ala	Ala	Arg	Val	Phe	Pro		Gly	Asn	Asp	Arg	Ala	Trp	Val	Ser	Leu	Thr	Ser	Ala	Gln									
Chymo A	Lys	Tyr	Asn	Ser	Leu	Thr	Ile			Asn	Asn	Asp	Ile	Thr	Leu	Leu	Lys	Leu	Ser	Thr	Ala	Ala	Ser	Phe	Ser	Gln	Thr	Val	Ser		
Elastase	Tyr	Trp	Asn	Thr	Asp	Asp	Val	Ala	Ala	Gly	Tyr	Asp	Ile	Ala	Leu	Leu	Arg	Leu	Ala	Gln	Ser	Val	Thr	Leu	Asn	Ser	Tyr	Val	Gln		
	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148		
SGPB				Ile	Pro	Lys	Asp	Gly	Thr	Val	Gly	Gly					Gln	Asp	Ile	Thr	Ser										
SGPA		Ala	Ala	Asn	Gly	Arg	Val	Tyr	Leu	Tyr	Asn	Gly				Ser	Tyr	Gln	Asp	Ile	Thr	Thr									
α-LP		Thr	Leu	Leu	Pro	Arg	Val	Ala	Asn	Gly	Ser		Ser			Phe	Val	Thr	Val	Arg	Gly										
Chymo A	Ala	Val	Cys	Leu	Pro	Ser	Ala	Ser	Asp	Asp	Phe	Ala	Ala	Gly	Thr	Thr	Cys	Val	Thr	Thr	Gly	Trp	Gly	Leu	Thr	Arg	Tyr	Thr	Asn		
Elastase	Leu	Gly	Val	Leu	Pro	Arg	Ala	Gly	Thr	Ile	Leu	Ala	Asn	Asn	Ser	Pro	Cys	Tyr	Ile	Thr	Gly	Trp	Gly	Leu	Thr	Arg		Thr	Asn		
	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	A	B	170	171	172	173	174	175	
SGPB							Ala	Ala	Asn	Ala	Thr	Val	Gly	Met	Ala				Val	Thr	Arg	Arg							Gly		
SGPA							Ala	Gly	Asn	Ala	Phe	Val	Gly	Gln	Ala				Val	Gln	Arg								Ser	Gly	
α-LP							Ser	Thr	Glu	Ala	Ala	Val	Gly	Ala	Ala				Val	Cys	Arg								Ser	Gly	
Chymo A	Ala	Asn	Thr	Pro	Asp	Arg	Leu	Gln	Gln	Ala	Ser	Leu	Pro	Leu	Leu	Ser	Asn	Thr	Asn	Cys	Lys	Lys				Tyr	Trp	Gly	Thr	Lys	
ELASTASE	Gly	Gln	Leu	Ala	Gln	Thr	Leu	Gln	Gln	Ala	Tyr	Leu	Pro	Thr	Val	Asp	Tyr	Ala	Ile	Cys	Ser	Ser	Ser	Ser	Ser	Tyr	Trp	Gly	Ser	Thr	
	176	177	178	179	180	181	182	183	184	185	186	186	186	186	186	186	186	186	186	186	186	186	186	186	186	186	186	186	186		
SGPB	Ser	Thr	Thr	Gly	Thr	His	Ser	Gly	Ser	Val	Thr	Ala	Leu	Asn	Ala	Thr	Val	Asn	Tyr	Gly	Gly	Gly	Asp	Val	Val	Tyr	Gly	Met	Ile		
SGPA	Ser	Thr	Thr	Gly	Leu	Arg	Ser	Gly	Ser	Val	Thr	Gly	Leu	Asn	Ala	Thr	Val	Asn	Tyr	Gly	Ser	Ser	Gly	Ile	Val	Tyr	Gly	Met	Ile		
α-LP	Arg	Thr	Thr	Gly	Tyr	Gln	Cys	Gly	Thr	Ile	Thr	Ala	Lys	Asn	Val	Thr	Ala	Asn	Tyr	Ala		Glu	Gly	Ala	Val	Arg	Gly	Leu	Thr		
Chymo A	Ile	Lys	Asp	Ala	Met	Ile	Cys	Ala	Gly	Ala	Ser																				
Elastase	Val	Lys	Asn	Ser	Met	Val	Cys	Ala	Gly	Gly	Asn																				
	187	188	A	188	189	190	191	A	B	192	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212
SGPB	Arg	Thr			Asn	Val	Cys	Ala	Glu	Pro	Gly	Asp	Ser	Gly	Gly	Pro	Leu	Tyr	Ser	Gly	Thr						Arg	Ala	Ile	Gly	Leu
SGPA	Gln	Thr			Asn	Val	Cys	Ala	Gln	Pro	Gly	Asp	Ser	Gly	Gly	Ser	Leu	Phe	Ala	Gly	Ser						Thr	Ala	Leu	Gly	Leu
α-LP	Gln	Gly			Asn	Ala	Cys	Met	Gly	Arg	Gly	Asp	Ser	Gly	Gly	Ser	Trp	Ile	Thr	Ser	Ala	Gly					Gln	Ala	Gln	Gly	Val
Chymo A	Gly	Val			Ser	Ser	Cys	Met				Gly	Asp	Ser	Gly	Gly	Pro	Leu	Val	Cys	Lys	Lys	Asn	Gly	Ala	Trp	Thr	Leu	Val	Gly	Ile
Elastase	Gly	Val	Arg	Ser	Gly	Cys	Gln					Gly	Asp	Ser	Gly	Gly	Pro	Leu	His	Cys	Leu	Val	Asn	Gly	Gln	Tyr	Ala	Val	His	Gly	Val

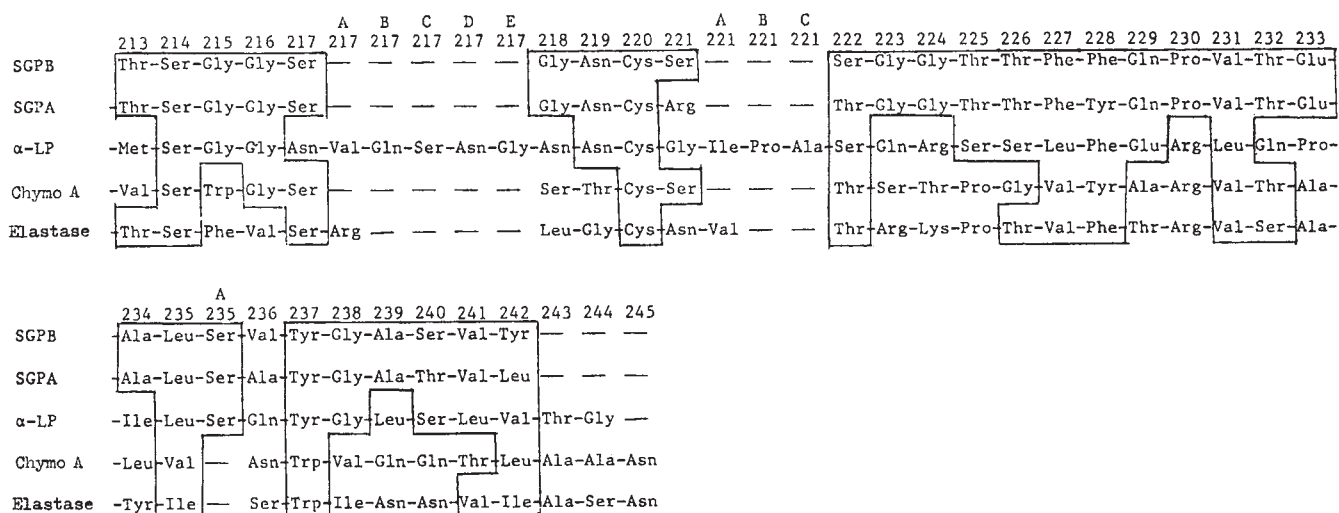


Fig. 2 Comparison of the amino acid sequences of SGPB, SGPA, α-lytic protease, bovine chymotrypsinogen A and porcine elastase. The sequence numbering is that of chymotrypsinogen A (ref. 19). Sequence homologies (chemically similar residues) are denoted by enclosing the amino acid residues within solid lines.

The disposition of the important residues of the catalytic triad is the same as that found for the other pancreatic serine proteases⁹⁻¹¹. Asp-102 is an internal residue and makes a close contact with His-57. The β-hydroxyl group of Ser-195 is also close to His-57, however we feel that the conformation in this region is different in minor respects to that in the active enzyme. The pH optimum is 7.0–8.0 for SGPB and as our study was done at pH 4.2 the enzyme is inactive. There is electron density close to the β-hydroxyl group of Ser-195 which we have tentatively assigned to two phosphate ions that interact with Ser-195 and a segment of the main chain between residues 216 and 217. There is a sulphate binding site observed at low pH in the active site region of one of the molecules of α-chymotrypsin^{21,22} and similar to the present phosphate binding site in SGPB. There is also a sulphate anion bound to the active site in elastase at pH 5.0 and this is replaced by two water molecules as the pH is raised to 8.5 (ref. 23). Serine protease inactivation at low pH may well derive in part from this anion binding in the active site region.

Polypeptide chain conformation

Figure 4 shows in diagrammatic fashion the path of the polypeptide chain. This view corresponds approximately to the standard view of the pancreatic enzymes depicting the molecule from a vantage point over the active site region. The polypeptide chain is folded to produce two hydrophobic cores with the active site located at their junction in a manner similar to that found in the pancreatic proteases. The first core which includes residues His-57 and Asp-102 is made up by folding the first 80 residues (16–128) in an extended conformation producing four major loops. The path taken by the chain in this first section of the molecule is similar to the conformation of the main chain in the mammalian proteases, with some notable exceptions.

The amino-terminal residue Ile-16 is not involved in an internal ion-pair interaction with Asp-194. Figure 4 shows that the amino terminus is on the surface of the enzyme with the *sec*-butyl side chain contributing to the first hydrophobic core and the amino group involved in close contact (possibly ion-pair interaction) with the β-carboxyl group of Asp-126. The chain length from the amino terminus to the 1/2 Cys-42 is much shorter in the microbial enzymes than in the pancreatic enzymes (Fig. 2). McLachlan and Shotton¹⁵ pointed out that

this short chain length was not consistent with the conformation of the polypeptide backbone in this region being identical in the two enzyme families. They did, however, suggest that with a minor conformational change the N-terminal amino group of α-lytic protease could form an ion pair with Asp-194. That this is not the case in the microbial enzymes is indicated by the present model of SGPB and was suggested earlier by the chemical work of Kaplan and Dugas²⁴, who showed that N-acetylation of the α-amino group in α-lytic protease had no effect on its enzymatic activity.

From the 1/2 Cys-42 the main chain traverses the molecule forming the so-called histidine loop before returning to complete the disulphide bridge at 1/2 Cys-58. This segment of the chain has four additional residues, 48A–48D, which are accommodated at the distal end of the histidine loop. Each of the three microbial enzymes SGPA, SGPB and α-lytic protease has these four extra residues.

Figure 2 shows that between residues 65 and 84 a large segment of the main chain of the pancreatic proteases is missing in SGPA and α-lytic protease, whereas only seven residues are deleted in SGPB. This region corresponds to the uranyl loop of α-chymotrypsin²⁵. In SGPA and α-lytic protease this loop is most certainly missing; however, Figure 4 shows that SGPB has a loop that corresponds to the uranyl loop of the pancreatic proteases albeit seven residues shorter.

The aspartate loop, so named because it contains the catalytically important Asp-102, makes up the fourth major loop in the first half of the molecule. The chain in SGPA and SGPB is five amino acids shorter than the chains of the mammalian enzymes and α-lytic protease. The important aromatic side chain (residue 94 which is Phe in the three microbial enzymes) is, however, present and in the SGPB structure serves partially to protect the Asp-102, His-57 interaction from exposure to the solvent. Those residues that are not present in SGPA and SGPB (95–99 inclusive) would form the distal end of the aspartate loop which also serves to shield the His-57, Asp-102 interaction in the mammalian enzymes.

Figure 2 shows that many deletions and insertions are required to produce some semblance of sequence homology between the mammalian enzymes and the microbial proteases in the region 112–191. Olsen *et al.*¹² noted that this region of α-lytic protease shows little homology with the pancreatic enzymes and that the sequence alignment for this section has been rather arbitrary.

Indeed in the microbial enzymes the autolysis loop is missing, but the polypeptide chain follows a path different from that taken in the mammalian proteases (Fig. 4). At residue 125, the only lysine residue of SGPB, the ϵ -NH₃⁺ group makes an ion pair with the α -carboxyl group of the terminal tyrosine-242. It is interesting that both the C terminus and the N terminus of SGPB are involved in ion-pair interactions with the neighbouring residues Lys-125 and Asp-126, respectively. Both SGPA and α -lytic protease have only the basic residue (Arg) at position 125 which may have some influence on the relative stabilities of SGPA and SGPB to denaturing conditions²⁶, although the unusual stability of SGPB to denaturants should be investigated more fully.

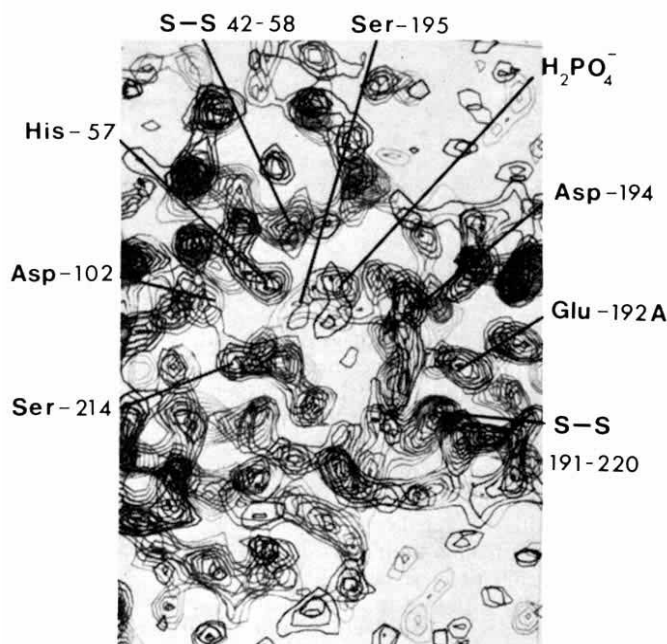


Fig. 3 Six sections (1-Å spacing) of the 2.8-Å electron density map of the SGPB molecule in the region of the active site. The residues that make up the active site are labelled. The two disulphide bridges 42-58 and 191-220 are also designated on these sections. The view of the molecule in these sections corresponds approximately to that shown in Fig. 4.

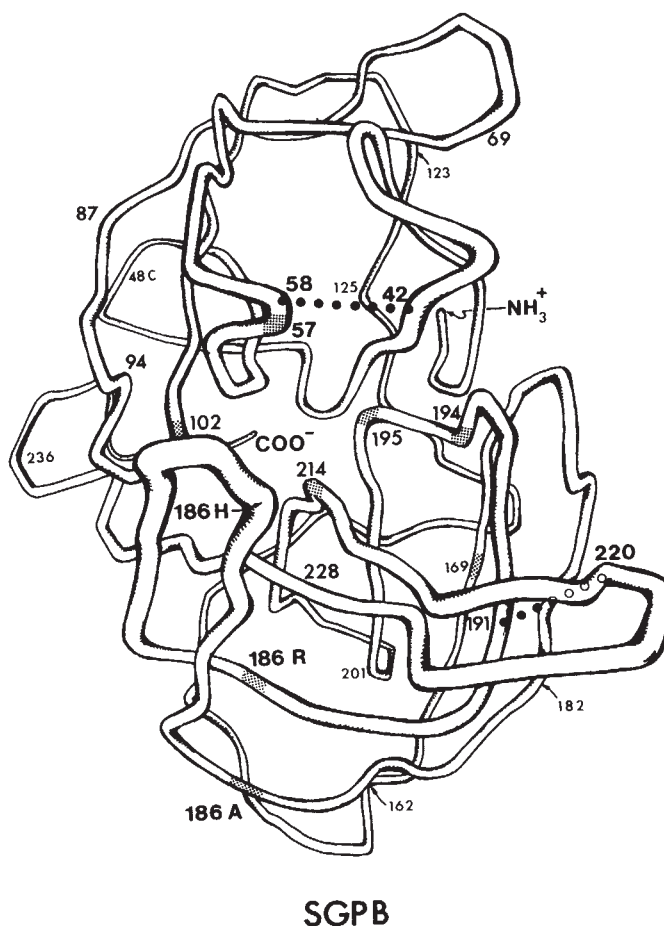
Two other segments of the polypeptide chain in SGPB in the region 123-191 are profoundly different in their locations relative to the tertiary structures of the pancreatic enzymes. The first is that segment of chain that is analogous to the methionine loop in the pancreatic enzymes (residues 168-182). The conformation of the methionine loop in SGPB is such that it occupies a volume in the SGPB molecule that corresponds to the autolysis loop of the pancreatic enzymes. This important structural difference places the side chain of a basic residue, Arg-169, in the appropriate orientation for the internally buried, positively charged guanidinium group to make an internal salt bridge with the carboxylate of Asp-194 (Fig. 4). In view of the methionine loop conformation observed for SGPB it seems unlikely that the microbial enzymes have a zymogen precursor. The enzymes must be catalytically active immediately the folding process has been completed after their synthesis. It could have been predicted from the results of Kaplan and Dugas²⁴ that the positive charge in this crucial ion pair must originate from some other portion of the molecule, but the residue providing the positive charge would have been impossible to predict with confidence. Even though the primary function of this basic residue at position 169 has been lost

through the evolution of the pancreatic serine proteases the basic nature of this residue is conserved in all serine proteases except elastase²⁷.

The other major difference in polypeptide chain conformation occurs at position 186 in the sequence of the microbial enzymes where there are 18 additional residues in SGPA and SGPB, and 17 in α -lytic protease. Clearly these residues do not have a counterpart in the pancreatic proteases. Therefore McLachlan and Shotton postulated that they formed an external loop (termed the compensating extra loop) which would substitute for the residues in the missing autolysis loop. As Fig. 4 shows, residues 186A-186R are not involved with providing a hydrophobic pocket that serves to bury the amino terminus, but rather they are in an extended β loop located on the opposite side of the molecule near the aspartate loop and this extra loop functions as a shield for the Asp-102, His-57 interaction from solvent. The five residues that are missing in the aspartate loop of SGPA and SGPB occur at a point where the loop bends back on itself in an antiparallel β -sheet conformation. These extra residues (95-99) in the pancreatic serine proteases occupy a volume that also shields the Asp-102, His-57 interaction from solvent (namely the hydrophobic residues at position 99). Therefore it appears that the microbial enzymes accomplish this shielding function in a rather extravagant fashion using an extra loop of 17 or 18 amino acid residues to do the task that five residues perform for the pancreatic serine proteases.

From the second disulphide bridge 1/2 Cys-191 to 1/2 Cys-220 the conformation of the polypeptide chain in SGPB

Fig. 4 A diagrammatic representation of the backbone conformation of the polypeptide chain from the 2.8-Å electron density map of SGPB. The labelled regions refer to the residue numbers of Fig. 2. The stippled segments correspond to approximate α -carbon positions for the designated residues. The disulphide bridges 42-58 and 191-220 are indicated.



closely resembles that in the pancreatic family. The residues involved in forming the specificity depression as well as the residues forming the serine loop are in this latter segment of the molecule. To form the serine loop the polypeptide chain traverses the central portion of the molecule and bends back on itself in a manner similar to the histidine loop conformation. From residue 214 to residue 228 there is a large loop of anti-parallel β -pleated sheet structure before the chain emerges from under the extra loop and completes the only two turns of α helix in the whole molecule, residues 231–238. This conformation of the chain is directly homologous to the main chain folding in the pancreatic enzymes. In SGPB, however, the final four amino acids are in an extended conformation and the ultimate residue, Tyr-242, has its aromatic side chain tucked back up into the hydrophobic region between the two central cores.

Active site and primary specificity site

The folding of the polypeptide chain of SGPB produces an active site region between the two hydrophobic cores in the enzyme that qualitatively resembles those active site regions in the pancreatic serine proteases. The amino acid side chains that are involved in the catalytic mechanism, that is, Asp-102, His-57, Ser-195 and Asp-194 are all similarly disposed in these two enzyme families and the catalytic triad is conserved as expected. A second serine residue, Ser-214, occupies a position that places its β -hydroxyl group within hydrogen bond distance of Asp-102 (Fig. 3). The importance of this second serine at position 214 has been pointed out by Kraut *et al.*²⁸ and its functional role in the catalytic mechanism is further substantiated by the study reported here. A much greater degree of homology than shown by McLachlan and Shotton¹⁵ is attained by having a three-residue deletion at position 205 in the α -lytic sequence as indicated in Fig. 2, which correctly places a serine residue at position 214.

The primary binding site of a pipsyl (*p*-iodobenzene sulphonyl) inhibitor on the SGPB molecule was described in the 4.5-Å resolution structural study¹⁷. The present 2.8-Å resolution map shows that this site is made up primarily of two short segments of main chain, residues 192–192B and residues 215–218. There is no evidence for a hydrophobic binding pocket or tosyl hole as seen in the α -chymotrypsin structure^{25,29}, however, in SGPB the binding site is a depression on the enzyme surface. The broad specificity of SGPB³⁰ is consistent with this binding site. The fact that SGPB will also hydrolyse bonds on the carboxyl side of basic residues may be due to the involvement of Glu-192A in this binding. With the second segment of the binding site made up chiefly of glycine residues, the whole binding region involves principally polypeptide backbone and has a shape that would accommodate large hydrophobic side chains¹⁷.

The binding pocket of α -lytic protease deserves comment. The revised alignment presented in Fig. 2 and similar to that proposed by Johnson and Smillie¹³ inserts a pentapeptide 217A–217E in this region. The α -lytic enzyme has a specificity similar to that of elastase and differs from the α -chymotrypsin specificity in that it does not cleave polypeptide chains on the carboxyl side of aromatic side chains. Clearly the extra five residues present in α -lytic protease would alter this region of the specificity depression from that seen in the SGPB structure, thus explaining the different specificities of these two enzymes.

SGPA has a broader but essentially similar specificity to that of SGPB³¹. Cleavage of the insulin B chain by SGPA occurs on the carboxyl side of Leu, Tyr, Phe, Arg and to a lesser degree Glu and Val. This similar specificity to SGPB and the fact that the sequence homologies between SGPA and SGPB are so great in the region of the substrate binding site, indicate that these two enzymes have almost identical tertiary structures in this region.

Evolutionary inferences

Although there are large differences in chain length (186 amino acids in SGPB compared with ~240 for the pancreatic enzymes) and relatively little in the way of sequence homology (Table 1) the overall tertiary structures are similar. Those sections of polypeptide chain which are almost similar, the histidine loop, the aspartate loop, the serine loop and the specificity loop (214–227) provide the probable structural core of the serine proteases. This similarity of structure provides a strong case for the divergent evolution of these serine proteases and demonstrates that this class of enzyme is very ancient and had an ancestor common to the mammals and bacteria.

The tertiary structural differences between these enzymes from such phylogenetically distant organisms also provide information on the evolution of protein structure. The evolution of catalytically inactive zymogens by multicellular organisms is a result of the necessity for the organism to protect itself from auto-digestion. McLachlan³² has suggested a piecemeal growth mechanism for the insertion or deletion of single amino acids into surface loops of an already biologically active protein in such a way that the activity is conserved. This mechanism allows for the addition (or deletion) of surface loops and for the concomitant improvement (or alteration) in biological activity. The structural core is conserved in going from the bacterial to the pancreatic enzymes; however, new stretches of chain have been added (in particular the N terminus and aspartate loop are lengthened, and the autolysis loop added) and other loops have been deleted (the extra compensating loop is lost after the acquisition of additional residues in the aspartate loop) or the function altered or lost (methionine loop).

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Regulation of cell cycle stage-specific transcription of histone genes from chromatin by non-histone chromosomal proteins

Gary Stein, William Park, Cary Thrall, Rusty Mans & Janet Stein

Department of Biochemistry, University of Florida, Gainesville, Florida 32610

RNA transcripts from chromatin of S phase but not G₁ cells contain histone-specific sequences. Chromatin reconstituted with S phase non-histone chromosomal proteins transcribes histone messenger RNA sequences whereas chromatin reconstituted with G₁ non-histone proteins does not. These results suggest that transcription of histone genes is regulated during the cell cycle and that non-histone proteins have a key role in this regulation.

SEVERAL lines of evidence suggest that non-histone chromosomal proteins are important in the regulation of gene expression in general¹⁻⁷, and specifically in the control of transcription during the cell cycle^{8,9}. Variations in the composition and metabolism of non-histone chromosomal proteins throughout the cell cycle are consistent with a regulatory function for these macromolecules¹⁰⁻¹⁷. More direct evidence that non-histone chromosomal proteins are regulatory macromolecules within this context comes from a series of chromatin reconstitution studies which demonstrate that non-histone chromosomal proteins are responsible for the increased transcriptional activity of S phase compared with mitotic chromatin^{8,9}.

In HeLa cells the synthesis of histones is confined to the S phase of the cell cycle¹⁸⁻²¹, and *in vitro* translation data have suggested that the messenger RNAs (mRNAs) for these basic chromosomal polypeptides are associated with a specific class of polysomes only at this time¹⁹⁻²³. We have added adenylic acid residues to the 3'-OH termini of histone mRNAs, and using RNA-dependent DNA polymerase, synthesised a histone complementary DNA (cDNA)²⁴. Using this high resolution probe, we have demonstrated that, consistent with the *in vitro* translation data, histone mRNA is associated with polyribosomes only during the S phase²⁵. In the present studies the histone cDNA was used to demonstrate that transcription *in vitro* of histone mRNA sequences is restricted to the S phase of the cell cycle and that non-histone chromosomal proteins are responsible for regulating the transcription from chromatin of those regions of the genome which contain the information for the synthesis of histones.

Characterisation of histone complementary DNA

To detect the presence of histone-specific RNA sequences, a single-stranded DNA complementary to histone mRNAs was synthesised using RNA-dependent DNA polymerase as described previously²⁴. 7-12S RNA was isolated from the polysomes of S phase HeLa S₃ cells and poly(A)-containing RNAs were removed by oligo(dT)-cellulose chromatography. The remaining 7-12S RNAs directed the synthesis of all five classes of histones in a cell-free protein synthesising system derived from wheat germ. Poly(A) (an average of 35 AMP residues) was added to the 3'-OH termini of the histone mRNAs with an ATP-polynucleotidyltransferase isolated from maize seedlings. The isolation and properties of this enzyme have been reported²⁶. The polyadenylated mRNAs were then transcribed with RNA-dependent DNA polymerase from

Rous sarcoma virus using dT₁₀ as a primer. The mean sedimentation coefficient of the cDNA in alkaline sucrose was 6.1S which corresponds to a size of approximately 400 nucleotides^{27,28}. The kinetics of hybridisation of the cDNA probe to histone mRNA isolated from polysomes of S phase HeLa S₃ cells were measured to assess the purity of the template RNA and cDNA (Fig. 1). The reaction proceeds with a C_0t_1 of 1.7×10^{-2} (see also double reciprocal plot³⁰ of the data, Fig. 1 inset). Since the molar concentration of the nucleotide sequences in solution determines the rate of hybridisation, comparison of the C_0t_1 of the histone mRNA to that of a kinetic standard such as globin mRNA (complexity of 1,200 bases³¹ and C_0t_1 in similar conditions of 3.8×10^{-3})³² yields a calculated sequence complexity of approximately 5,400 bases which is two times greater than expected for the total complexity of the five histone messages. This is, however, within the range of variation found for the rate of RNA-DNA hybridisation^{30,33-35}. When cDNA and histone mRNA are hybridised in the absence of formamide at 75 °C (data not shown), the C_0t_1 is 5×10^{-3} . From the C_0t_1 of globin mRNA in these

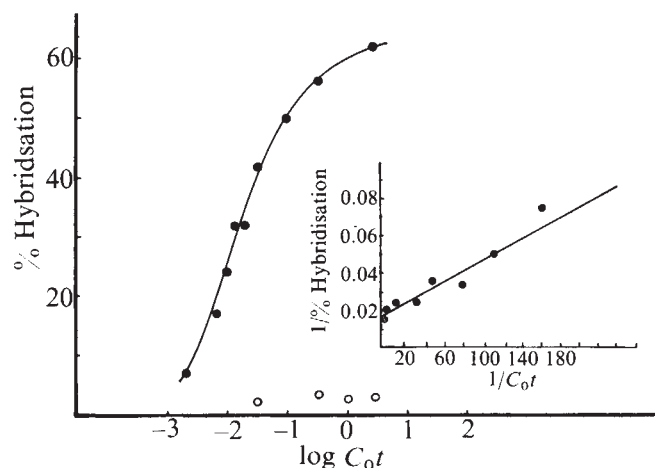


Fig. 1 Kinetics of annealing of histone cDNA to histone messenger RNA isolated from the polysomes of S phase HeLa S₃ cells. 400 c.p.m. of ³H-cDNA (27,000 d.p.m. ng⁻¹) were annealed at 52 °C in sealed glass capillary tubes in a volume of 15 µl containing 50% formamide, 0.5 M NaCl, 25 mM HEPES (pH 7.0) and 1 mM EDTA with either 0.03 or 0.19 µg of histone mRNA in the presence of 3.75 µg of *E. coli* RNA as carrier (●) or with 3.75 µg of *E. coli* RNA in identical conditions (○). The reaction mixtures were assayed for hybrid formation using fraction IV, single-strand specific S₁ nuclease isolated from *Aspergillus oryzae*²⁹. Each sample was incubated for 20 min in 2.0 ml of 30 mM sodium acetate, 0.3 M NaCl, 1 mM ZnSO₄, 5% glycerol (pH 4.6), containing S₁ nuclease at a concentration sufficient to degrade at least 95% of the single-stranded nucleic acids present. The amount of radioactive DNA resistant to digestion was determined by trichloroacetic acid precipitation. No background values have been subtracted. C_0t = mol ribonucleotides s l⁻¹. Inset: Double reciprocal plot of the kinetics of annealing of histone cDNA and histone mRNA. A background of 3% has been subtracted from each point.

conditions ($C_0t_i = 2.0 \times 10^{-3}$)^{36,37} the sequence complexity of histone mRNA is estimated to be 3,000 bases. When the probe is annealed with *Escherichia coli* RNA in either of the above conditions, no significant level of hybrid formation is detected. The low level of S_1 nuclease resistant TCA-precipitable radioactivity may be accounted for by a limited amount (3%) of ^3H -cDNA which is not digested by the enzyme in the incubation conditions used. Thermal denaturation curves of the histone mRNA-cDNA hybrids exhibit a single transition with a T_m of 65 °C in 50% formamide-0.5 M NaCl-25 mM HEPES (pH 7.0)-1 mM EDTA and 95 °C in 0.5 M NaCl-25 mM HEPES (pH 7.0)-1 mM EDTA. These T_m values are consistent with the reported base composition of histone mRNA of 54% G-C (ref. 38).

Additional evidence for specificity of the histone cDNA is its ability to form hybrids with total polysomal RNA isolated from intact S phase HeLa cells ($C_0t_i \sim 1.8$) and its lack of hybrid formation with G_1 polysomal RNA²⁵. From these findings it is reasonable to conclude that ribosomal (5S, 18S and 28S) and transfer RNA complementary sequences are not present in the histone cDNA. Furthermore, the polysomal RNA isolated from HeLa cells in which histone and DNA synthesis have been blocked by cytosine arabinoside does not form hybrids with the histone cDNA. These results are consistent with data from several laboratories which indicate that histone mRNA is not present on the polyribosomes of HeLa cells treated with inhibitors of DNA synthesis^{19,23,39-42} and additionally rule out the possibility that the cDNA contains detectable amounts of sequences complementary to other S-phase specific, non-polyadenylated RNAs which have been reported to be insensitive to cytosine arabinoside²³.

Transcription of histone-specific sequences from G_1 and S phase chromatin

Evidence has indicated that in HeLa cells histone synthesis is restricted to the S phase of the cell cycle and that the synthesis of these basic chromosomal polypeptides is dependent on concomitant DNA replication¹⁸⁻²⁰. It has also been shown that mRNAs for histones are associated with polysomes during S phase and not during other periods of the cell cycle^{19-21,23,25}. One might therefore anticipate that the synthesis of mRNAs for histones is restricted to the period of DNA replication. Direct evidence for this contention is lacking, however. Therefore, as an initial approach to studying histone gene transcription we examined the abilities of chromatin from G_1 and S phase cells to transcribe histone-specific sequences *in vitro*.

The degree to which *in vitro* RNA transcripts from G_1 and S phase chromatin hybridise to the histone ^3H -cDNA was determined. Figure 2 shows the polyacrylamide gel electrophoretic distribution of RNAs synthesised from HeLa S_3 cell chromatin with *E. coli* RNA polymerase. More than 90% of the RNAs synthesised migrated in the 4-14S region of the gels reflecting the synthesis of RNA molecules which are 75-1,500 nucleotides long. The data in Fig. 3 clearly indicate that although histone-specific sequences are present in the transcripts from S phase chromatin, such sequences are not detected in the transcripts from chromatin of G_1 phase cells. Specifically, the ^3H -cDNA hybridises to S phase chromatin transcripts with a C_0t_i of 2.1×10^{-1} , whereas no hybridisation above the background level of 3% was detected with G_1 transcripts. The extent of hybridisation of transcripts from chromatin of exponentially growing HeLa cells ($C_0t_i = 4 \times 10^{-1}$) was approximately 50% that observed for S phase transcripts (approximately 50% of an exponentially growing population of HeLa cells are undergoing DNA synthesis at any given time) suggesting that thymidine synchronisation used for obtaining S phase cells does not significantly alter transcription of histone mRNA sequences. It should be noted that the T_m (65 °C in 50% formamide-0.5 M NaCl-25 mM HEPES (pH 7.0)-1 mM EDTA and 95 °C in 0.5 M NaCl-25 mM HEPES (pH 7.0)-1 mM EDTA) of the hybrids formed between the ^3H -cDNA and S phase chromatin

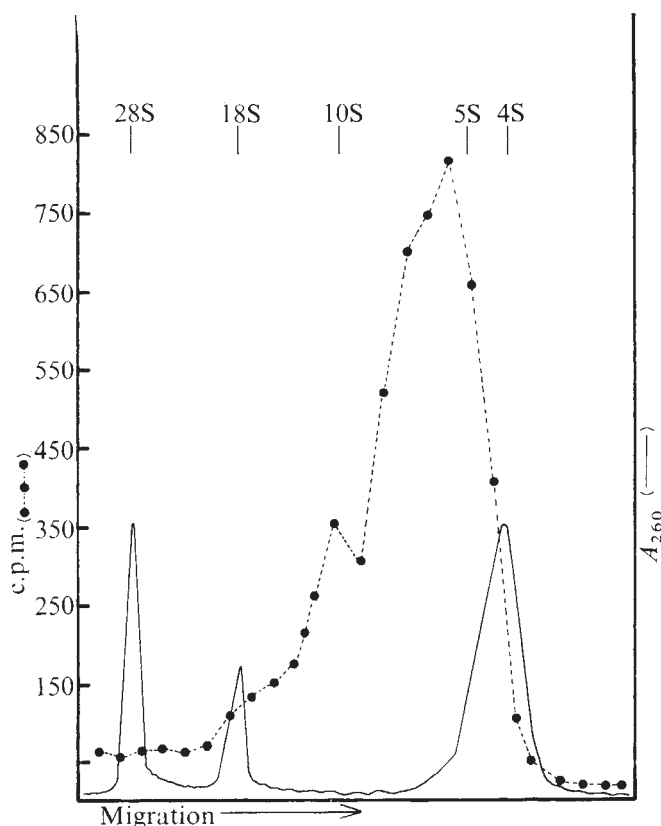


Fig. 2 Polyacrylamide gel electrophoresis of RNAs transcribed from HeLa S_3 cell chromatin in the presence of ^{14}C -ATP (0.4 $\mu\text{Ci ml}^{-1}$). Electrophoresis was carried out according to the method of Schochetman and Perry⁴³ in the presence of 4S, 18S and 28S RNAs as internal markers. RNA was transcribed using Fraction V *E. coli* RNA polymerase prepared according to the method of Berg *et al.*⁴⁴. Transcription was carried out for 70 min at 37 °C in a Dounce homogeniser fitted with a wide clearance pestle, and the reaction mixture was periodically homogenised to maintain chromatin solubility. The incubation mixture in a final volume of 10 ml contained: 0.04 M Tris (pH 8.0); 4 mM MgCl_2 ; 1 mM MnCl_2 ; 0.02 mM EDTA; 0.008% β -mercaptoethanol; 0.4 mM each of ATP, CTP, UTP, and GTP; 150 $\mu\text{g ml}^{-1}$ of DNA as chromatin; and 600 U RNA polymerase. RNA was extracted as follows. The reaction was brought to a concentration of 1% SDS-0.1 M NaCl-0.01 M sodium acetate-1 mM EDTA (pH 5.4) and incubated at 37 °C for 15 min. Following two extractions with equal volumes of phenol and chloroform-isoamyl alcohol (24:1, v/v) and two extractions with chloroform-isoamyl alcohol, nucleic acids were precipitated with 3 volumes of ethanol. The pellet was resuspended in 10 mM Tris-0.1 M NaCl-5 mM MgCl_2 (pH 7.4) containing 40 $\mu\text{g ml}^{-1}$ of DNase I and incubated at 37 °C for 60 min. Following one extraction with phenol-chloroform-isoamyl alcohol and two with chloroform-isoamyl alcohol, the aqueous phase containing the RNA transcripts was chromatographed on Sephadex G-50 fine and eluted with 50 mM Tris-0.1 M NaCl-1 mM EDTA (pH 7.2). RNA was precipitated with 3 volumes of ethanol. For hybridisation analysis the RNA was resuspended in 25 mM HEPES-0.5 M NaCl-1 mM EDTA, pH 7.0.

transcripts is identical to the T_m of the ^3H -cDNA-histone mRNA hybrids. From a comparison of the C_0t_i values of the S phase chromatin transcript-cDNA and histone mRNA-cDNA hybridisation reactions it can be estimated that 8% of the S phase transcripts are histone-specific sequences. The figure of 8% is based on the assumption that the histone mRNA preparation consists entirely of histone mRNA sequences. The presence of other sequences in the preparation would require a corresponding reduction in this figure.

To rule out the possibility that endogenous RNAs associated with S phase chromatin at least in part account for the formation of hybrids with histone cDNA, the following approach was pursued. S phase chromatin was placed in the transcription mixture without RNA polymerase and an amount of *E. coli*

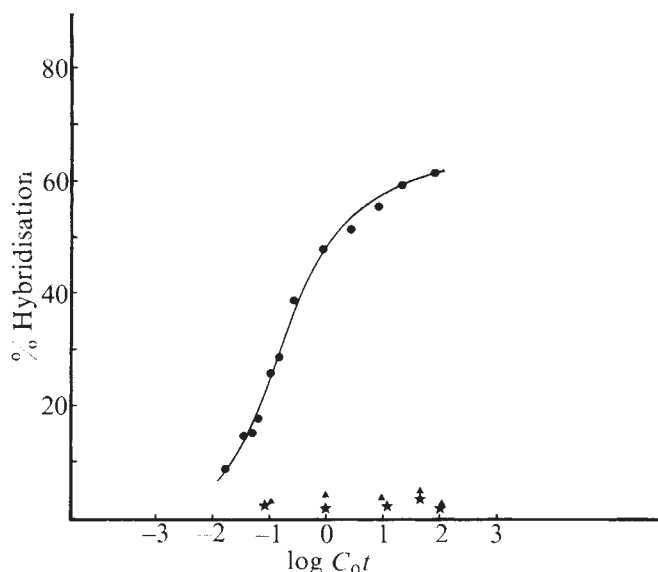


Fig. 3 Kinetics of annealing of histone cDNA to *in vitro* transcripts of chromatin from G_1 and S phase HeLa S_3 cells. 400 c.p.m. of ^3H -cDNA (27,000 d.p.m. ng^{-1}) were annealed at 52°C to either 0.15 or 1.5 μg of RNA transcripts from G_1 ($\blacktriangle$) or S phase ($\bullet$) chromatin. 400 c.p.m. of cDNA were also annealed to 1.5 μg of *E. coli* RNA isolated in the presence of S phase chromatin ($\star$). *E. coli* RNA was included in each reaction mixture so that the final amount of RNA was 3.75 μg . Exponentially growing HeLa S_3 cells were maintained in suspension culture in Joklik-modified Eagle's MEM supplemented with 3.5% each of calf and foetal calf serum. Cells were synchronised as described previously¹⁰. S phase cells were obtained by synchronisation with 2 cycles of 2 mM thymidine block. 3 h after release from the second thymidine block cells were collected; at this time 98% of the cells were in S phase as determined by autoradiographic assessment of ^3H -thymidine labelled nuclei. G_1 cells were obtained 3 h after selective detachment of mitotic cells from semiconfluent monolayers. 97% of the cells were in the G_1 phase of the cell cycle; incorporation of ^3H -thymidine into nuclei could not be detected autoradiographically, reflecting the complete absence of S phase cells. Nuclei were obtained by washing cells 4 times in 80 volumes of Earle's balanced salt solution and lysing the cells in 80 volumes of 80 mM NaCl–20 mM EDTA–1% Triton X-100 (pH 7.2). The nuclei were washed three times with the lysing medium and then twice with 0.15 M NaCl–0.01 M Tris (pH 8.0). Nuclei isolated in this manner are free of cytoplasmic material when examined by phase contrast and electron microscopy. Lysis of nuclei was achieved by suspending the nuclear pellet in triple glass-distilled water with several strokes of a wide-clearance Dounce homogeniser. The chromatin was allowed to swell at 4°C for 30 min, pelleted at 20,000g for 15 min, resuspended in distilled water, and again pelleted at 20,000g.

scription during the cell cycle, the evidence is of a correlative nature. To examine directly the involvement of non-histone chromosomal proteins in the control of cell cycle stage-specific gene readout, we pursued the following approach. Chromatin isolated from G_1 and S phase cells was first dissociated, in 3 M NaCl–5 urea–0.01 M Tris (pH 8.3), and the DNA was pelleted at 150,000g for 36 h. Proteins were fractionated into histone and non-histone chromosomal protein fractions by the QAE–Sephadex method of Gilmour and Paul⁴⁵. Chromatin was reconstituted by the gradient dialysis procedure of Bekhor *et al.*⁴⁶. The details of these methods⁸ and evidence for fidelity of chromatin reconstitution^{46–48} have been reported. DNA was prepared by the method of Marmur⁴⁹, treated with pancreatic ribonuclease A (50 $\mu\text{g ml}^{-1}$ for 30 min at 37°C) and Pronase (50 $\mu\text{g ml}^{-1}$ for 2 h at 37°C), and then extracted twice with phenol and chloroform–isoamyl alcohol before use.

In vitro RNA transcripts from chromatin reconstituted with G_1 non-histone chromosomal proteins and from chromatin reconstituted with S phase non-histone chromosomal proteins were annealed with histone ^3H -cDNA. Figure 4 shows that RNA transcripts from chromatin reconstituted with S phase non-histone chromosomal proteins hybridise with histone cDNA although those from chromatin reconstituted with G_1 non-histone chromosomal proteins do not exhibit a significant degree of hybrid formation. It should be emphasised that the kinetics and extent of hybridisation with the cDNA are the same for transcripts of native S phase chromatin and transcripts of chromatin reconstituted with S phase non-histone chromosomal proteins (Fig. 4). Furthermore, the amounts of RNA transcribed and the recoveries during isolation of these transcripts from the native and reconstituted chromatin preparations are essentially identical. These results imply a functional role for non-histone chromosomal proteins in regulating the availability of histone sequences for transcription from chromatin and, taken together with the polysomal data presented previously²⁵, suggest that, in intact cells, regulation of histone gene expression during the cell cycle may reside, at least in part, at the transcriptional level. Such a regulatory role for non-histone chromosomal proteins is in agreement with results from several laboratories which have indicated that these

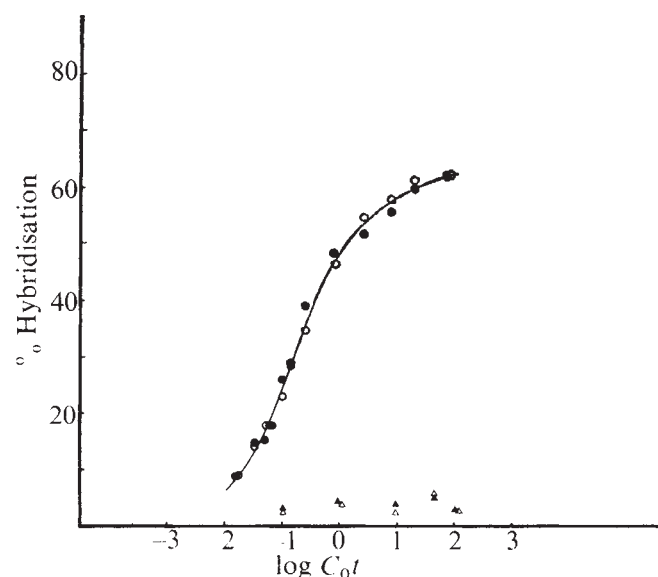


Fig. 4 Kinetics of annealing of histone cDNA to *in vitro* transcripts from native and reconstituted chromatin. 400 c.p.m. of ^3H -cDNA were annealed at 52°C with either 0.15 μg or 1.5 μg of RNA transcripts from chromatin reconstituted with S phase non-histone chromosomal proteins and DNA and histones pooled from G_1 and S phase cells ($\circ$), chromatin reconstituted with G_1 non-histone chromosomal proteins and DNA and histones pooled from G_1 and S phase cells ($\triangle$), native S phase chromatin ($\bullet$) and native G_1 chromatin ($\blacktriangle$). *E. coli* RNA was included in each reaction mixture so that the final amount of RNA was 3.75 μg .

RNA equivalent to the amount of RNA transcribed from S phase chromatin was added. RNA was immediately extracted by the same procedure used for the isolation of *in vitro* transcripts. When this control RNA was annealed with the cDNA no significant level of hybridisation was observed (Fig. 3). Additionally, RNA isolated from S phase chromatin in the absence of carrier shows no hybrid formation with the cDNA. These results establish that endogenous histone-specific sequences associated with S phase chromatin are not contributing significantly to the hybridisation observed with S phase *in vitro* transcripts. The findings presented so far are consistent with the argument that the genetic sequences which code for the synthesis of histones are available for transcription from chromatin during the S phase of the cell cycle but not during G_1 .

Regulation of histone-specific sequences by non-histone chromosomal proteins

Although evidence has been presented which strongly suggests that among the non-histone chromosomal proteins are macromolecules which are responsible for the regulation of tran-

proteins are responsible for the tissue-specific transcription of globin genes^{2,36,50}.

Although it seems that among the non-histone chromosomal proteins are macromolecules which regulate the transient readout of histone genes during the cell cycle, the regulatory elements involved have yet to be defined. It is not clear whether the non-histone chromosomal proteins which render histone sequences transcribable during S phase, are synthesised at the onset of DNA replication or whether they are pre-existing proteins which are enzymatically modified at this time. Post-translational modifications of non-histone chromosomal proteins, particularly phosphorylation, have been implicated as having a key role in dictating structural as well as transcriptional properties of the genome. Furthermore, it remains to be established whether control is of a positive or negative nature. Since the genetic sequences for histone mRNAs in HeLa cells are reiterated⁴², the possibility arises that a single cell may contain multiple copies of the proteins which control the expression of histone genes. This system may therefore be readily amenable to the isolation of such regulatory proteins.

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Theory and practice for studies of peptides by ¹⁵N nuclear magnetic resonance at natural abundance: gramicidin S

G. E. Hawkes & E. W. Randall

Department of Chemistry, Queen Mary College, Mile End Road, London E1 4NS, UK

C. H. Bradley

Bruker-Physik AG, 7501 Karlsruhe-Forchheim, West Germany

The low-abundance isotope of nitrogen, ¹⁵N, is now accessible to study by the latest high resolution nuclear magnetic resonance techniques. Structure and motion in polypeptides of moderate size may now be usefully investigated in this way.

THE difficulties in observing magnetic resonance spectra (NMR) of the ¹⁵N nucleus at the natural abundance level (0.36%) are well established^{1–3}. Recent advances in technique^{4–6}, however, specifically the pulse-Fourier transform method with ¹H noise decoupling, have made it possible to obtain useful spectra. The initial studies on natural abundance ¹⁵N NMR, however, used neat liquid samples^{4,7} or highly concentrated solutions of low molecular weight material^{8,9}. Of course the low sensitivity has occasionally been elevated for ¹⁵N (as for other nuclei, such as ¹³C) by isotopic enrichment⁸. Labelling techniques then can add a new dimension to mechanistic studies, as witnessed by the nitrosation work on peptide

models, in which the fate of ¹⁵N-labelled nitrite ion is followed by NMR⁹.

The rewards of studying ¹⁵N NMR are first, that one observes a high resolution nucleus that may participate directly in fundamental chemical and biological processes (for example, acid-base equilibria, tautomerism, hydrogen bonding and coordination), and second, that the spectra of complex nitrogen-containing molecules, for example peptides, should be far simpler, and therefore easier to interpret than their ¹³C- or ¹H-spectra which can be over-rich in resonances.

Some general considerations for ¹⁵N NMR

The sensitivity of ¹⁵N in an NMR experiment at constant field compared with ¹³C (both isotopes at natural abundance) is about 1:30. Thus, to obtain comparable signal-to-noise ratios from experiments using signal averaging on the two species at similar total nitrogen and carbon concentrations would take about 900 times longer for ¹⁵N than for ¹³C, which is already a difficult nucleus. A combination of larger sample volumes (25 mm outer diameter tubes) and a spectrometer

Table 1 ^{15}N Chemical shifts of some amino acid methyl ester hydrochlorides in water

R	$\delta^{15}\text{N}^*$	R	$\delta^{15}\text{N}^*$
H (Glycine)	10.0	HSCH_2 (Cysteine)	19.4
CH_3 (Alanine)	23.0	$\text{CH}_2\text{SCH}_2\text{CH}_2$ (Methionine)	19.6
$(\text{CH}_3)_2\text{CH}$ (Valine)	18.3	$\text{CH}_2\text{SCH}_2\text{CH}_2$ (Methionine)	21.2
$(\text{CH}_3)_2\text{CHCH}_2$ (Leucine)	21.8	(Proline)	34.7
$\text{CH}_3\text{CH}_2\text{CH}(\text{CH}_3)$ (Isoleucine)	18.9	$\text{CH}_3\text{O}_2\text{CCH}_2$ (Methylaspartate)	19.3
$\text{C}_6\text{H}_5\text{CH}_2$ (Phenylalanine)	20.9	$\text{NH}_2=\text{C}(\text{NH}_2)\text{NH}(\text{CH}_2)_3^{\dagger}$ (Arginine . HCl)	21.6
$p\text{-HO} \cdot \text{C}_6\text{H}_4\text{CH}_2$ (Tyrosine)	20.4	$\text{NH}_3(\text{CH}_2)_4^{\ddagger}$ (Lysine . HCl)	21.9
HOCH_2 (Serine)	15.8	(Histidine . HCl) §	22.2

The chemical shifts are included in ref. 21.

Solutions were 3–4 M in H_2O , pH adjusted in the range 0.6–1.5.

* Chemical shifts in p.p.m. (± 0.3) downfield from NH_4^+ resonance of 5 M $^{15}\text{NH}_4^{15}\text{NO}_3$ in 2 N HNO_3 (see ref. 22 for this choice).⁴ The NO_3^- resonance is 354.1 p.p.m. to low field.

† Guanidine resonances at 43.3 (NH_2) and 54.8 (HN) p.p.m.

‡ Additional NH_3 resonance at 5.8 p.p.m.

§ Imidazole resonances at 144.2, 146.3 p.p.m.

with a higher operating magnetic field (4.2 T) may be used to bring lower sensitivities within range for either ^{13}C or ^{15}N provided the samples are not limited in amount. Sensitivity gains of between 15 and 20 which have been achieved for ^{15}N in this way are reported here. Sample concentrations may now be reduced and material of higher molecular weight may be studied. Additionally various new experiments have become accessible, which can give useful information about motional as well as structural characteristics. (A very elementary exposition of the basic NMR experiment is given in ref. 10.)

Lippmaa et al.¹¹ have found long (50–500-s) spin-lattice relaxation times (T_1) for ^{15}N in a range of small organic molecules. Where spectra are to be accumulated by repetitive pulse-Fourier transform methods, saturation of the resonances is then very easy, and can result in reduced signal intensity. This problem may be overcome by the addition of paramagnetic material to the sample⁶ to shorten T_1 , or by the use of long delays ($> T_1$) between pulses. We have now found that for larger molecules the problem of long T_1 s is not so serious (see below). (A useful introduction to relaxation times is ref. 12.)

The nuclear Overhauser effect (NOE) (reviewed in refs 12–14) is a well known intensity effect that occurs in double resonance experiments¹⁰. For ^{15}N , as for other nuclei, it can be defined as the ratio of the proton-decoupled ^{15}N signal intensity (I_D) to the proton-coupled intensity (I_0). If the motion of the molecules is fast enough (small correlation time, τ_c) then in the absence of exchange effects on T_1 , the NOE (denoted by $1 + \eta$) is given by

$$\frac{I_D}{I_0} = 1 + \eta = 1 + \frac{\gamma(^1\text{H})T_{1D}^{-1}}{2\gamma(^{15}\text{N})T_1^{-1}} \quad (1)$$

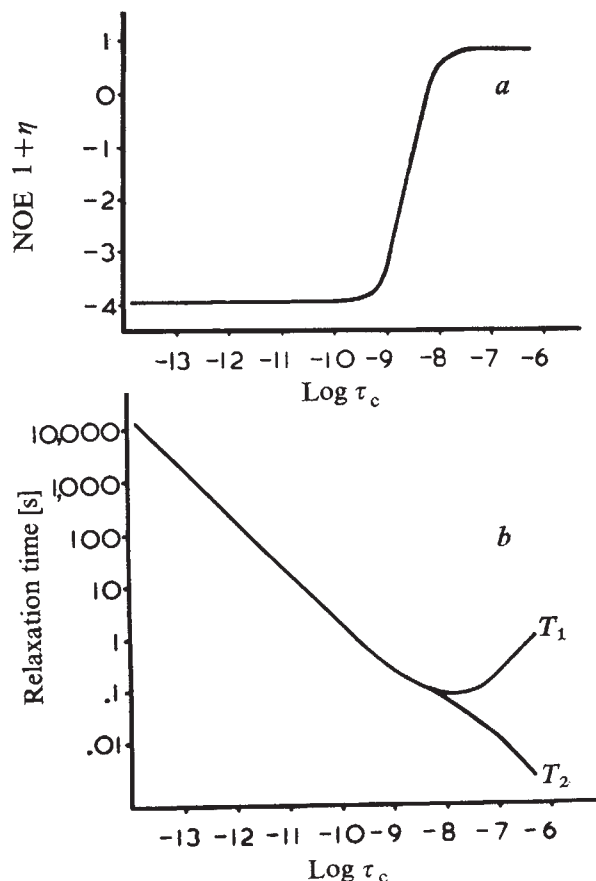
where T_{1D} is the ^{15}N – ^1H dipole–dipole contribution to the total ^{15}N relaxation time, T_1 . Since the signs of the magnetogyric ratios (γ) of ^{15}N and ^1H are opposite ($1 + \eta$) can be negative^{4,7}. The maximum possible NOE, which occurs for the 100% dipole–dipole case, is in fact -3.9 . An inverted absorption mode signal then results. Other mechanisms for relaxation

for example, spin-rotation, chemical shift anisotropy^{11,13,14} may, however, compete with the dipole–dipole (that is, $T_{1D} > T_1$) thus reducing the NOE. In unfavourable situations $1 + \eta \rightarrow 0$, and zero ^{15}N signal results⁷. Again, addition of paramagnetic material to the sample may be used, this time to provide a dominant electron–nucleus (^{15}N) relaxation mechanism⁶. Then $T_1 \ll T_{1D}$ giving $1 + \eta \rightarrow 1$. An alternative way of suppressing the NOE which has been used frequently for ^{13}C is the gated decoupling technique¹⁵. We report here its first use for proton-decoupled ^{15}N systems to give ^{15}N resonances decoupled but without NOE.

The strength of the dipole–dipole mechanism depends on the inverse sixth power of the ^{15}N – ^1H internuclear separation and so it is most efficient for ^{15}N with directly bonded protons. To obtain inverted absorption mode ^{15}N signals (that is a significant NOE), however, it is not necessary that the protons be directly bonded, as has been shown by Lichter and Roberts⁷ on nicotine and Katz *et al.*⁸ on pheophytin a. What is necessary is merely that other relaxation mechanisms for ^{15}N should not be significant relative to the dipole–dipole.

If the approximation of fast motion does not hold, however, reduced NOEs can still result even if the dipole–dipole mechanism dominates. Allerhand and Oldfield¹⁶ have shown this for ^{13}C – $\{^1\text{H}\}$ experiments. (I – $\{S\}$ signifies observation of the I spins with saturation of the S spin transitions.) We report a similar analysis for the ^{15}N – $\{^1\text{H}\}$ system (Fig. 1a). Figure 1b shows a plot of T_1 and T_2 for a single ^{15}N – ^1H interaction, with a magnetic field of 2.1 T and internuclear distance, $r = 1.03 \text{ \AA}$. The expected reduction in the NOE occurs also for ^{15}N , and most importantly the sign of the signal changes as τ_c changes. In the region of fast motion (characterised by the inequality, $\omega^2\tau_c^2 \ll 1$, where ω is the resonance frequency) the fully inverted signal is obtained. When, however, $\omega^2\tau_c^2 \gg 1$, the signal is no longer inverted and the NOE reaches a limiting value of $+0.88$. At intermediate values of τ_c (about 6×10^{-9} s) signal

Fig. 1 a, Theoretical maximum ^{15}N – $\{^1\text{H}\}$ NOE. ^{15}N at 9.12 MHz; b, ^{15}N – ^1H dipole–dipole relaxation time. ^{15}N at 9.12 MHz.



nulling occurs. This motional nulling is to be contrasted with the original nulling condition we mentioned: the competitive relaxation situation.

We have observed a third nulling situation when off-resonance single frequency decoupling¹⁰ is used. Such experiments have proved to be very useful in oversoming assignment difficulties, notably in ¹³C NMR¹⁷. This possibility, and even a fourth nulling condition resulting from the gated decoupling experiment, have recently been discussed briefly¹⁸.

Table 2 ¹⁵N chemical shifts of some N-formyl and N-acetyl amino-acids

R	R HO ₂ C-CH-NHCHO	R HO ₂ C-CH-NHCOCH ₃
	N-Formyl δ ¹⁵ N 3-4 M in DMSO*	N-Acetyl δ ¹⁵ N 1 M in DMSO
H (Glycine)	93.7	89.4
CH ₃ (Alanine)	108.7	104.4
(CH ₃) ₂ CH (Valine)	101.7	98.0
CH ₃ CH ₂ CH(CH ₃) (Isoleucine)	102.5	99.0
(CH ₃) ₂ CHCH ₂ (Leucine)	106.0	101.7
C ₆ H ₅ CH ₂ (Phenylalanine)	104.4	100.9
HSCH ₂ (Cysteine)	—	99.0
CH ₃ SCH ₂ CH ₂ (Methionine)	104.7	—
Proline	115.3† 113.7†	110.8‡ 114.0§ 114.6¶
β-Alanine	—	96.4

Chemical shifts in p.p.m. (±0.3) downfield from ¹⁵NH₄⁺ resonance of 5M ¹⁵NH₄⁺NO₃.

* These data are included in ref. 21.

† *cis* and *trans* isomers.

‡ Both isomers (unresolved at 9.12 MHz).

§ *trans* isomer in MeOH-d₄.

¶ *cis* isomer in MeOH-d₄.

Nitrogen is an atom which often participates directly in chemical exchange phenomena. The ¹⁵N spectrum of a nucleus exchanging between two or more chemically shifted sites may display resonances due to each particular environment (slow exchange), a single averaged resonance (fast exchange), or at intermediate exchange rates much broadened resonances which are difficult to observe. Even if the sites are equivalent, exchange may affect the spectrum by causing decoupling of ¹⁵N from an attached labile proton. Assignment can then not be made by observation of multiplicities.

Contrary to first assessments¹⁹, it should not be thought that the NOE of a nitrogen attached to a labile proton need necessarily be affected by the exchange, simply because the multiplicity is lost. In other words, the single upright resonances in single resonance spectra may be inverted by double irradiation of the sample. We have observed this behaviour for the amino resonances of viomycin sulphate, for which the amido portions exhibited normal multiplicities (unpublished results of L. F. Farnell, G.E.H. and E.W.R.). The original expectation of an exchange effect on the NOE came from the possibility that modulation of the scalar ¹⁵N-¹H interaction (which gives rise to the multiplicity) could affect *T*₁ and thus the NOE. A dominant mechanism of this sort could give a value 1+η = +10.86. Leipert and Noggle have shown, however, that even when the scalar relaxation is at a maximum it is most unlikely to dominate²⁰.

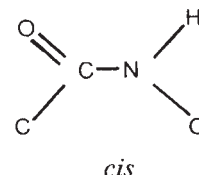
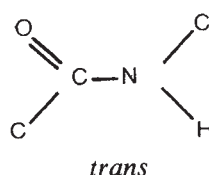
On the other hand, it is conceivable that exchange could affect the NOE by a new consideration. Suppose we have fast exchange of ¹⁵N between inequivalent sites, one can then obtain

the average NOE. Nulling could then occur if in the slow exchange limit the signals are opposite in sign.

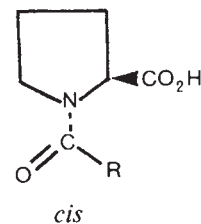
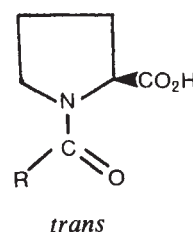
Applications to peptides

As a necessary preliminary to studying larger peptides we have investigated the types of structural information which may be obtained by ¹⁵N NMR work at natural abundance on small model systems; moreover systems wherein complicating effects of chemical exchange at nitrogen are minimal. Tables 1 and 2 show the ¹⁵N chemical shift data for some amino acid methyl ester hydrochlorides (some of which have been the subject of a preliminary communication⁶) and some N-acyl amino acids. The chemical shift variations which we have so far observed are due almost entirely to structural changes because medium effects have been shown to be small. For example a pH study on glycine methyl ester hydrochloride showed the ¹⁵N shift was insensitive to pH change in the range 0-4. Similarly the N-formyl derivatives (Table 2) showed a negligible shift with concentration in the range 1-4 M in DMSO, and for the N-acetyl derivatives the effect of changing the solvent from DMSO to methanol is to impart only a small (1.3±0.5 p.p.m.) downfield shift. The cause of this last small effect is probably the differing intermolecular hydrogen bonding situations in the two solvents.

For each example shown in Table 2, a single ¹⁵N resonance was generally observed and is due²³ to the *trans* amide form.



Resolution of the *cis* and *trans* resonances of N-acetyl L-proline in DMSO (about 71% *trans* in DMSO solution²⁴) was achieved only at the higher resonance frequency (18.24 MHz, 4.2 T) and not at 9.12 MHz (2.1 T). The shift difference was 0.3 p.p.m. with the *cis* signal being to higher field. This illustrates the second advantage, further to the sensitivity gain, of operating at higher frequency. For a solution in methanol-d₄ the solvent induced shift was sufficient to give resolved signals at 9.12 MHz: the signal for the *cis* isomer was 0.6 p.p.m. to low field.



The differential effects, amounting to about 3.5 p.p.m. (±0.3 p.p.m.), on ¹⁵N chemical shifts in amino acids by formylation and acetylation (Table 2) lead in theory to the possibility of obtaining sequence information for small peptides by ¹⁵N NMR. Table 3 shows the ¹⁵N chemical shifts of some dipeptide and a tripeptide N-acetyl derivatives, which were obtained to test this possibility. Consider first the series: AcGly, AcGly¹Gly², AcGlyAla, AcGlyLeu. The signal at about 89 p.p.m. for all must be due to Gly (Gly¹ for AcGly¹Gly²), and demonstrates that the introduction of the C-terminal amino acid unit has a negligible effect on the ¹⁵N shift. This is verified by the comparisons AcAla, AcAlaGly (Ala at about 104.5

Table 3 ^{15}N chemical shifts of some N-acetyl peptides

	Concentration* (M)	$\delta^{15}\text{N}^\dagger$	
Ac-Gly-Gly	1	89.1	84.0
Ac-Gly-Gly-Gly	0.8	89.7(1)‡	84.0(2)‡
Ac-Gly-L-Leu	1	89.1	96.4
Ac-L-Leu-Gly	1	102.2	84.6
Ac-Gly-L-Ala	1	89.1	98.8
Ac-L-Ala-Gly	1	104.7	83.5

*Solutions in DMSO-d_6 .† Chemical shifts in p.p.m. (± 0.3) downfield from NH_4^+ resonance of $5\text{M } ^{15}\text{NH}_4^+ ^{15}\text{NO}_3^-$.

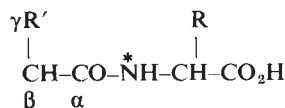
‡ Numbers in parentheses are relative signal intensities.

p.p.m.) and AcLeu, AcLeuGly (Leu at about 102 p.p.m.).

The introduction of an N-terminal amino acid unit does, however, shift the ^{15}N resonance of the C-terminal unit (in the dipeptides) away from the acetyl amino acid position as shown by the comparisons:

AcGly	AcGlyGly	AcAlaGly	AcLeuGly
89.4	84.0	83.5	84.6 p.p.m.

The shift seems to be insensitive, however, to the structure of the N-terminal amino acid unit. This is reasonable, since a change in the N-terminal unit is a γ or δ effect across a carbonyl group:



Thus the ^{15}N chemical shift of glycine, for example, in a peptide chain would be expected to be about 84 p.p.m. ± 0.5 p.p.m. at least in DMSO solution. This is verified by the shifts for N-acetyltryglycine:

Ac-Gly-Gly-Gly
89.7 84.0 84.0 p.p.m.

In the same way that glycine in a peptide gives a ^{15}N shift about 5.4 ± 0.5 p.p.m. upfield from N-acetyltryglycine, so also the alanine in AcGlyAla gives a shift 5.6 p.p.m. upfield from the value for AcAla, and leucine in AcGlyLeu is 5.3 p.p.m. upfield from AcLeu. Thus, the theoretical promise of getting sequence information is in practice not realised for these derivatives in DMSO solution. The relative numbers and the nature of the peptide components are, however, easily obtained.

^{15}N study of gramicidin S

The naturally occurring, antibiotic, cyclic decapeptide gramicidin S, molecular weight 1,120, has the primary structure cyclo-(D-Phe-L-Pro-L-Val-L-Orn-L-Leu) $_2$ and has been studied extensively as a model for proteins by both proton²⁶ and ^{13}C NMR²⁶⁻²⁸. Its relatively high solubility in both methanol and DMSO makes it a suitable substrate for testing the applicability of ^{15}N NMR at the natural abundance level to higher molecular weight material. Spectra obtained at 2.1 T are shown in Fig. 2

Table 4 ^{15}N chemical shifts for gramicidin S

Solvent	Concentration	$\delta^{15}\text{N}^*$			
Methanol	0.2 M	11.5	97.4	105.2	106.7
		115.6			
DMSO-methanol (4:1)†	0.25 M	14.2	93.4	103.3	104.7
		106.5	114.0		
DMSO	0.3 M	16.1			

* Chemical shifts in p.p.m. (± 0.3) downfield from NH_4^+ resonance of $5\text{M } ^{15}\text{NH}_4^+ ^{15}\text{NO}_3^-$.

† Sample prepared by dilution of 0.3 M solution in DMSO by methanol.

(the chemical shifts are summarised in Table 4) and illustrate the excellent resolution obtainable in spite of the relatively long accumulation times required. The backbone amide nitrogens resonate in the region 93–116 p.p.m. whereas the side-chain $-\text{NH}_2$ of ornithine is substantially to higher field. That a spectrum in DMSO solution (Fig. 2c) showed no amide nitrogen resonances is noteworthy. It is the first example of nulling of ^{15}N resonances by the restricted motional effects outlined above.

To confirm this proposal, ^{13}C NMR spectra of gramicidin S at 22.6 MHz were recorded, in various solvent and concentration conditions with ^1H decoupling, and the half-widths of various well resolved resonances were measured (Table 5). The full resonance width at half height ($\Delta\nu_1$ Hz) is related to the spin-spin relaxation rate, T_2 , by $1/T_2 = \pi\Delta\nu_1$ (s^{-1}). From these data, effective correlation times (τ_c) for overall reorientation of gramicidin S were calculated (Table 5). The correlation times obtained for solutions 0.2 M in methanol and 0.3 M in

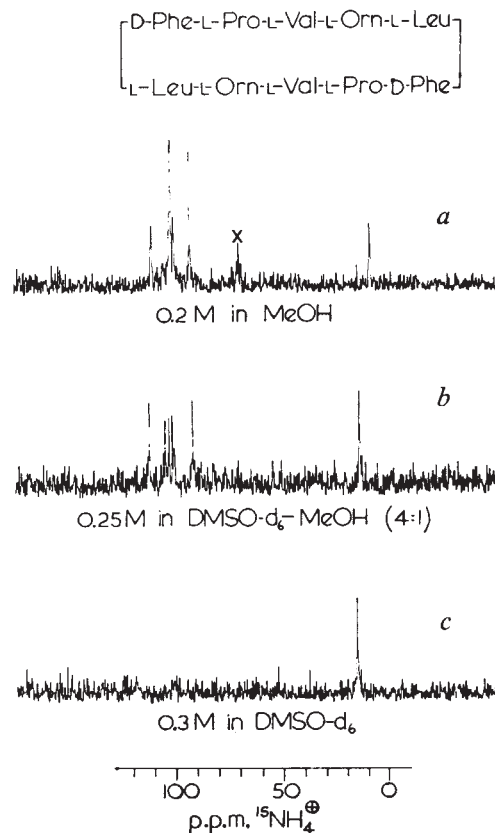


Fig. 2 ^1H Noise decoupled natural abundance ^{15}N NMR spectra (9.12 MHz) of gramicidin S. Average accumulation time per spectrum was about 36 h. These are magnitude spectra and thus contain no information on the sign or magnitude of the NOE. X marks the position of coherent noise from the deuterium lock.

DMSO were $(6 \pm 3) \times 10^{-10}$ s and $(5.4 \pm 0.8) \times 10^{-9}$ s, respectively. (The first value agrees with that obtained by Allerhand and Komoroski²⁷ from ^{13}C T_1 measurements on a more dilute solution of gramicidin S in methanol: $\tau_c = (3.3 \pm 0.2) \times 10^{-10}$ s.) Use of these data in Fig. 1 shows that the ^{15}N resonances from the 0.2 M solution in methanol must have a near maximum NOE ($1 + \eta = -3.9$ to -3.6), whereas those from the 0.3 M solution in DMSO are in the nulled region.

Further confirmation that the ^{15}N resonances from the solution of gramicidin in DMSO were nulled by the NOE was provided by two experiments at 18.2 MHz: a variable temperature experiment and a gated decoupling experiment. The ^1H decoupled spectrum of gramicidin (0.15 M in DMSO) at about 18 °C showed no amide resonances and a strong NH_2 reso-

Table 5 Average ^1H decoupled ^{13}C resonance half widths for gramicidin S

Carbons	0.2 M in methanol- d_4	0.23 M in DMSO- d_6 /methanol- d_4	0.23 M in DMSO- d_6	0.3 M in DMSO- d_6
Methyls	NR	3	4	6
Methine C	5	6	9	14
Carbonyl	2	2	2	4
$\tau_c \times 10^{10}$ s	6 ± 3	10 ± 5	22 ± 7	54 ± 8

Full line widths at half height (± 1.2 Hz) are estimated to include about 1 Hz instrumental broadening. NR, not fully resolved—overlapping resonances.

nance (Fig. 3b). An increase of sample temperature from 18 °C to 47 °C decreased the correlation time sufficiently to quench the nulling and give amido resonances showing a partial NOE, as can be seen in Fig. 3. In the gated decoupling experiment the conditions were chosen to yield decoupled resonances without NOE and the spectrum in Fig. 3b was thereby made to yield amido resonances opposite in phase to those obtained at 47 °C. These experiments were feasible only because of the increased sensitivity at 18.24 MHz.

In every ^{15}N spectrum (at 9.12 and 18.24 MHz) the side chain NH_2 resonance of ornithine was observed. Here, the significantly greater mobility of the side chain²⁷ relative to the backbone of the molecule ensures a sufficiently short correlation time to preclude signal nulling.

From the shift data presented above (Tables 2 and 3) it is possible to make a full assignment for the amide nitrogen resonances of gramicidin S. To a first approximation the data in Table 4 predict the amide nitrogen resonance of a given amino acid in a peptide chain to be independent of its position in the chain but to be influenced strongly by its substituents at the α carbon. We have not observed the amide nitrogen of ornithine in a peptide chain elsewhere but its shift can be calculated approximately⁵. Thus the relative amide nitrogen chemical

Fig. 3 ^1H Noise decoupled natural abundance ^{15}N NMR spectra (18.24 MHz) of gramicidin S (0.18 M in DMSO). Average accumulation time per spectrum was about 40 min. Both spectra are out of phase by 180 °, that is, signals are inverted by the NOE. a, 47 °C; b, 18 °C.

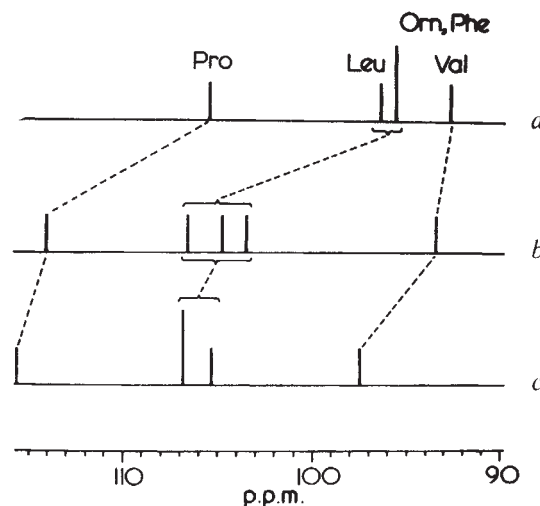
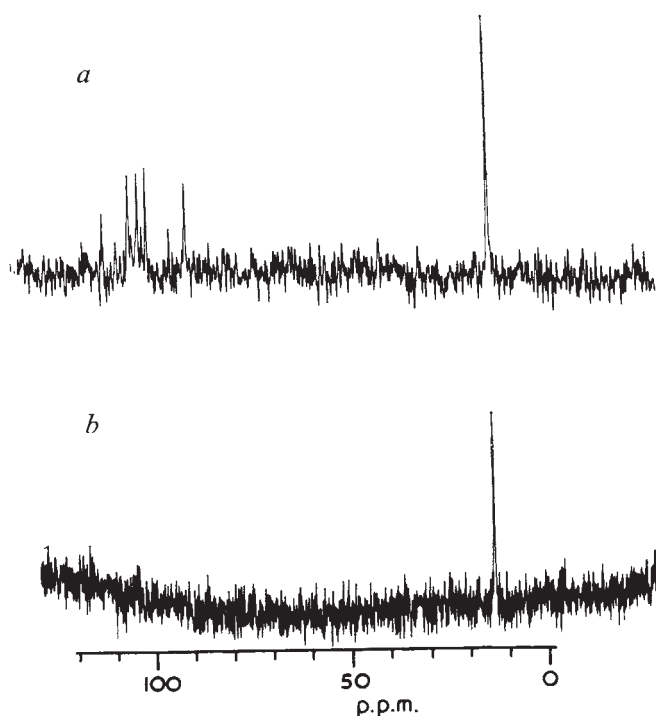
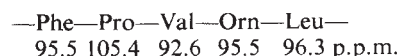


Fig. 4 Correlation diagram of amide nitrogen resonances from gramicidin S. a, Predicted from model dipeptides (see text); b, observed from solution 0.23 M in DMSO; c, observed from solution 0.2 M in methanol.

shifts of gramicidin S are predicted as the acetyl amino acid shifts (Table 2) minus 5.4 p.p.m. (Table 3). (This actually gives the amide nitrogen resonance as the C-terminal unit of an N-acetyl dipeptide.)



Although the patterns of observed and predicted chemical shifts are similar, the predicted ones are each substantially to higher field than the observed shifts as shown by the correlation diagram (Fig. 4). The reason for this is not yet clear, but it should be remembered that the predictions are from model dipeptides which include free carboxylic acid groups, whereas gramicidin S has side chain $-\text{NH}_2$ groups. A second possible source for the displacement in Fig. 4 is the unknown effect of constraining the peptide in a cyclic structure and introducing intramolecular hydrogen bonding between leucine and valine²⁵. Each of these effects is the subject of a continuing study.

For our study the amino acid and small peptide derivatives were prepared by standard methods. We used gramicidin J from *Bacillus brevis* (nagano) (Sigma) which has the same chemical structure as gramicidin S. ^{15}N NMR spectra were obtained from the Bruker HFX-90 (^{15}N at 9.12 MHz) and Bruker WH-180 (^{15}N at 18.24 MHz) instruments operating in the pulse-Fourier transform mode with ^1H noise decoupling. Samples were contained in tubes of outer diameter 10, 13 or 15 mm (HFX-90) and 25 mm (WH-180). Deuterium in the solvent or, in the case of aqueous (H_2O) or methanol (CH_3OH) solutions, deuterium oxide in a concentric 5-mm tube, provided the field-frequency stabilisation signal. D_2O and CD_3OD were not normally used as solvent in order to prevent exchange of deuterium for labile protons in the solute. Typical spectral accumulation times (HFX-90) were 5–10 h for 1-M solutions of amino acid derivatives with pulse flip angles about 30 °, and pulses were repeated every 0.4 s. The higher magnetic field, larger sample volume and quadrature detection system of the WH-180 instrument provided an estimated increase in signal-to-noise of about 18:1 for a single pulse experiment, or a saving in time corresponding to a factor of about 300 for spectral accumulation, when compared with the HFX-90 even with 15-mm tubes.

The accuracy of the measured ^{15}N chemical shifts was limited by the digitisation of the transformed spectrum (2.4 Hz per point) and was about 0.3 p.p.m. for the HFX-90 and 0.15 p.p.m. for the WH-180. ^{15}N Chemical shifts were referenced to the

ammonium ion resonance from an external sample of $^{15}\text{NH}_4^+$ $^{15}\text{NO}_3^-$ 5 M in 2-N nitric acid.

^{13}C Spectra at 22.6 MHz were obtained with the HFX-90 instrument. Sample temperatures for the HFX-90 instrument were about 35 °C, and for the WH-180 instrument are estimated to be accurate to ± 3 °C.

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letters to nature

Clustering of faint blue objects

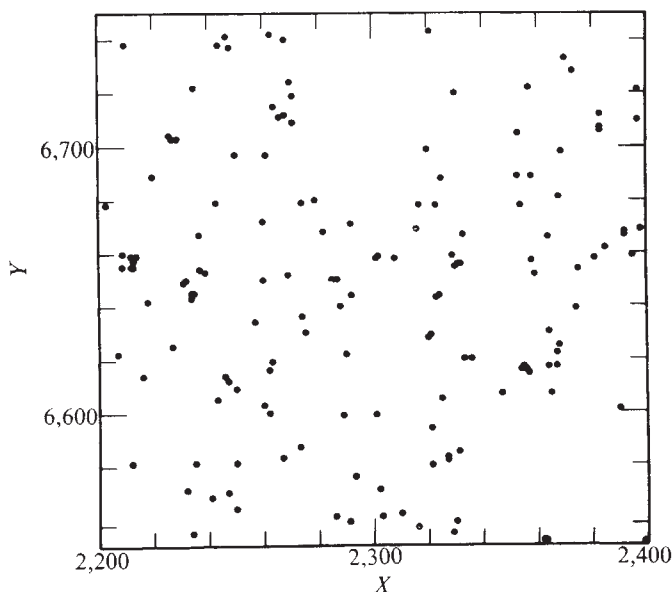
PHOTOGRAPHS taken with the UK 48-inch Schmidt Telescope at Siding Spring, Australia using hypersensitised Kodak IIIaJ emulsions, record objects as faint as magnitude 23 in exposures of an hour. Most galaxies on the photographs are very faint and at, say 22 mag probably have redshifts $Z \sim 0.5$. At such large redshifts the K term is responsible for considerable reddening ($B-V \simeq +2$ mag) and it may perhaps not be expected that such blue sensitive emulsions would show much increase in the number of faint galaxies recorded as the limiting magnitude increases beyond about 21 mag. The number of objects does, however, seem to continue to increase significantly with limiting blue magnitude and the question therefore arises as to whether or not a substantial number of faint images are blue and if so, what objects do they represent and how are they distributed?

To examine this question a IIIaJ photograph of a region near the south Galactic pole has been compared with a red-sensitive photograph of the same region. Both plates reach very faint magnitudes and when viewed in a Zeiss comparator most images are similar on both plates—that is to say, are of neutral apparent colour. The outer parts of some relatively bright spirals are noticeably blue, the inner parts neutral. Among the faint objects (which are the vast majority) a significant minority, several per cent, are noticeably blue, and a smaller minority are noticeably red.

The positions of noticeably blue objects in an area 22 arc min square were measured by one of us (V.C.R.) and the distribution is shown on Fig. 1. There are 168 objects. Only objects for which images appeared on both red and blue plates were recorded in order to avoid the accidental inclusion of plate faults. The criterion of 'blueness' by which they were chosen in this way is of course arbitrary and subjective. They represent about 10% of all the images in that area of the photographs: that is to say they are the bluest 10% of the objects. At the magnification employed, $\times 13$, almost all the images appear stellar. Comparison with a similar IIIaJ photograph of the cluster Kron 3 near the SMC in which M. F. Walker has determined electronographic magnitudes to $B = 22.7$ shows that many, perhaps most, of the objects are $B = 22$ or fainter and the great majority are fainter than $B = 21$.

It is immediately apparent from Fig. 1 that the apparent surface distribution is non-random. There is a tendency to clustering and particularly for the occurrence of pairs. This is confirmed by Fig. 2, which shows the frequency distribution of the separations of each object from its nearest neighbour (histogram $O(V.C.R.)$). A Poisson distribution with the same average separation (45'') is shown, $P(45,168)$, for comparison. If the objects were uniformly distributed the average separation would be 100'' and so the observed distribution represents a concentration by a factor of five. It has a sharp peak at a much smaller separation than the average, at 10–20'', and a second broad maximum around 70''. It is fairly well represented by the sum of two Poisson distributions $P(20,102;77,66)$ in which two thirds of the objects have a mean separation of 20'' and the remaining third a mean separation of 77''. The first of these

Fig. 1 The apparent distribution of faint blue objects as measured by V.C.R.



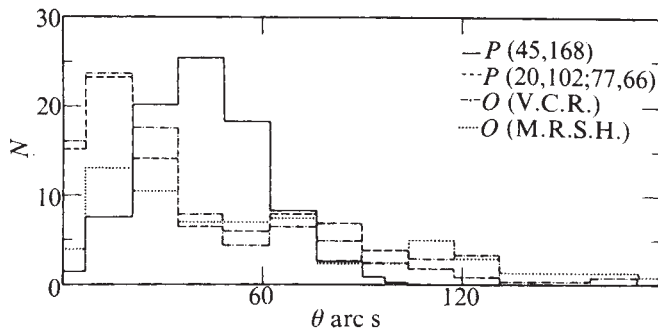


Fig. 2 N is the number of faint blue objects per unit of $7''$ in θ . θ is the angular separation of each from its nearest neighbour. O (V.C.R.) is the distribution measured by V.C.R. corresponding to Fig. 1; O (M.R.S.H.) that measured by M.R.S.H. corresponding to Fig. 3. The latter's threshold was chosen more blue than the former's and thus the total number of objects is smaller (73 as against 168). $P(45,168)$ is a Poisson distribution with the same mean θ (45) and total N (168) as O (V.C.R.). $P(20,102; 77,66)$ is the sum of two Poisson distributions with mean θ 20 and $77''$ and total numbers 102 and 66 respectively.

distributions represents the preponderant number of pairs evident on Fig. 1.

In order to check the validity of the data, especially in view of its element of subjectivity, and to provide data for another region, M.R.S.H. similarly measured the positions of the bluest objects in a second region of the same size but separated by several degrees from the first. His result is shown on Fig. 3. The number of objects is smaller, 73 against 168, and it appears from a cross check by the two of us that M.R.S.H. set his

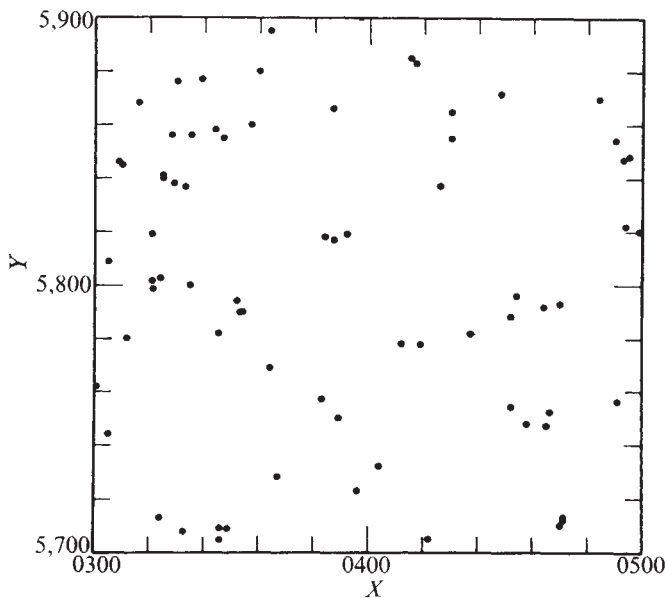


Fig. 3 The apparent distribution of faint blue objects as measured by M.R.S.H. in a region several arc degrees away from Fig. 1.

criterion at a bluer level than V.C.R.; thus Fig. 3 represents the distribution of the bluest 5% of the images. The same effects are again immediately apparent, however—some clustering and a preponderance of pairs. This impression is also confirmed by the histogram O (M.R.S.H.) in Fig. 2.

The non-random nature of the distribution makes it very unlikely that the objects are faint blue stars in the Galactic halo; they are almost certainly extragalactic. The faintness and star-like quality of the images indicate that they are distant, perhaps very distant, and compact. The blueness suggests that they are

not ordinary galaxies but lie in the range from very blue compact galaxies to quasars. Whatever they are the high degree of clustering, and especially the preponderance of pairs, appears to be significant. Taken together with a recent indication of an increase in the degree of clustering of galaxies with distance¹ this may be further direct evidence of an evolving universe.

M. R. S. HAWKINS
V. C. REDDISH

Department of Astronomy,
University of Edinburgh,
Royal Observatory, Edinburgh 9, UK

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Possible local variation of the Hubble constant in van den Bergh's calibration of Sc-type galaxies

IN an important series of papers for the determination of the Hubble constant, Sandage and Tammann¹⁻⁶ have concluded that the Universe is expanding in a remarkably uniform and isotropic way, with Hubble constant (H) $55 \pm 5 \text{ km s}^{-1} \text{ Mpc}^{-1}$. If true, this result is evidently crucial for all future cosmological models. As a consequence it should be tested and analysed in all possible ways. Their low H value is evidently based on their recalibration of the absolute magnitudes of the various luminosity classes of Sc-type galaxies based on two samples of nearby, and one sample of distant galaxies (Tables 3 and 4, ref. 5, and the Table, ref. 6).

Here we discuss some consequences of Sandage and Tammann's specific choice of absolute magnitudes, and to compare them with the calibration of van den Bergh⁷, which had been generally accepted in the literature.

Two preliminary remarks should be made here. First, starting from the data and calibration of Sandage and Tammann, de Vaucouleurs has analysed possible variations of the redshift distribution with supergalactic longitude and latitude, and discovered significant deviations from isotropy. Second, if one calculates H using only the Sc I galaxies, one does indeed

Table 1 Recalibration of Sandage and Tammann's data on sources in local supergalaxy anticentre direction using van den Bergh's absolute magnitudes

Sc classes	M_{ST}	M_{vdb}	$\alpha = H_{vdb}/H_{ST}$
I	-21.25	-20.0	1.778
I-II	-20.74	-19.7	1.614
II	-20.23	-19.4	1.466
II-III	-19.72	-18.9	1.459
III	-19.21	-18.3	1.521

find the same H at all distances, although Sandage and Tammann's unbiased data for Sc II anticentre galaxies (Tables 3 and 4, ref. 5), yield $\langle H \rangle = 77.6 \pm 6.0 \text{ km s}^{-1} \text{ Mpc}^{-1}$, which differs significantly from the result for Sc I anticentre galaxies (Table 2) $\langle H \rangle = 61.4 \pm 6.8 \text{ km s}^{-1} \text{ Mpc}^{-1}$ so that $\Delta H = 19.2 \pm 8.1 \text{ km s}^{-1} \text{ Mpc}^{-1}$ (Student's $t = 2.53$).

If, then, one accepts de Vaucouleurs' result (unpublished), that the overall motion of the local supergalaxy contributes significantly to the redshift distribution, one should concentrate discussion of the possible consequences for H determination of a recalibration of Sandage and Tammann's samples with van den Bergh's absolute magnitudes, on sources located in the anticentre of the local supergalaxy direction.

We do this in Table 1. Column 1 shows the Sc classes used in Sandage and Tammann's determination of H ; columns 2

Table 2 Comparison of values of H obtained for the new calibration, for the two samples described in the text

Sc-type	H_{ST} km s ⁻¹ Mpc ⁻¹	H_{vdb} km s ⁻¹ Mpc ⁻¹	H_{ST} km s ⁻¹ Mpc ⁻¹	H_{vdb} km s ⁻¹ Mpc ⁻¹
Sc I	61.40 ± 6.84	109.16 ± 12.16	51.73 ± 2.72	91.98 ± 4.83
Sc I-II	67.83 ± 9.59	109.48 ± 15.5		
Sc II	77.61 ± 5.99	113.70 ± 8.82		

and 3, the values of the corresponding absolute magnitudes used by them and by van den Bergh, column 4, the numerical factors by which the $H = v/r$ values of Sandage and Tammann should be multiplied, to pass to the corresponding values in van den Bergh's calibration, that is α in $H_{vdb} = \alpha H_{ST}$.

The calculations have been performed on all the unbiased anticentre galaxies in refs 5 and 6. For nearby galaxies we have used exactly the limit proposed by Sandage and Tammann to eliminate a possible bias, that is, for those with values of $\log v \leq 3.3$. The same has been done for distant Sc I galaxies—we have restricted the calculation (as prescribed by Sandage and Tammann) to the 40 anticentre objects with $\log v \leq 3.92$.

The results are summarised in Table 2. Column 1 shows the Sc classes analysed; columns 2 and 3, the corresponding H values obtained in calibrations by Sandage and Tammann and van den Bergh for the sample of 7 Sc I, 4 Sc I-II and 8 Sc II nearby unbiased anticentre galaxies. Columns 4 and 5 contain the respective H values for the unbiased sample of 40 distant anticentre Sc I galaxies.

We derive three conclusions from Table 2. The first is that Sandage and Tammann's calibration yields significant differences in the $\langle H \rangle$ values obtained with different types of nearby Sc galaxies—a fact difficult to reconcile with their own assumptions. The second is that van den Bergh's calibration, though possibly open to criticism, does not present this inconvenience, and yields a consistent $\langle H \rangle$ value for all classes of nearby Sc galaxies: $\langle H \rangle = 111.2 \pm 6.3$ km s⁻¹ Mpc⁻¹, for 19 objects. The third is that this latter value differs significantly from the distant Sc $\langle H \rangle$ value (that is, $\langle H \rangle = 92 \pm 5$ km s⁻¹ Mpc⁻¹) since we have $\Delta H = 19.2 \pm 8.1$ km s⁻¹ Mpc⁻¹.

If one accepts van den Bergh's calibration, this evidently yields a statistically significant decrease of H with distance, since for the hypothesis $H(\text{nearby}) = H(\text{distant})$, $t = 2.38$ ($P < 0.05$). Finally it should be noted that the existence of such a variation of H is independently supported by very recent determinations of the distance D of 29 Sc-type galaxies (12 of which belong to the preceding Sandage-Tammann sample) made on the 21-cm line at the Nançay Radiotelescope by Durand⁸ and Bottinelli *et al.* (unpublished). Indeed, if one divides Durand's result into two groups: the first with 15 objects at $D \leq 30$ Mpc and the second with 14 objects at $D > 30$ Mpc, one obtains $\langle H \rangle_1 = 95.2 \pm 11.0$ and $\langle H \rangle_2 = 63.5 \pm 5.4$ km s⁻¹ Mpc⁻¹.

Obviously, this possible variation of H with distance can be correlated with the angular anisotropy of H observed by Rubin, Ford and Rubin⁹, which has been confirmed on various types of source¹⁰. Independently of any specific interpretation (such as a variation of the redshift of photons passing through the radiation field of distant galactic clusters¹⁰, this conflict between the results of calibrations on the same sample by Sandage and Tammann and van den Bergh shows that further observational work needs to be done on this calibration problem.

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G. LE DENMAT
M. MOLES
J. P. VIGIER

Institut Henri Poincaré

J. L. NIETO

Institut d'Astrophysique,
Paris, France

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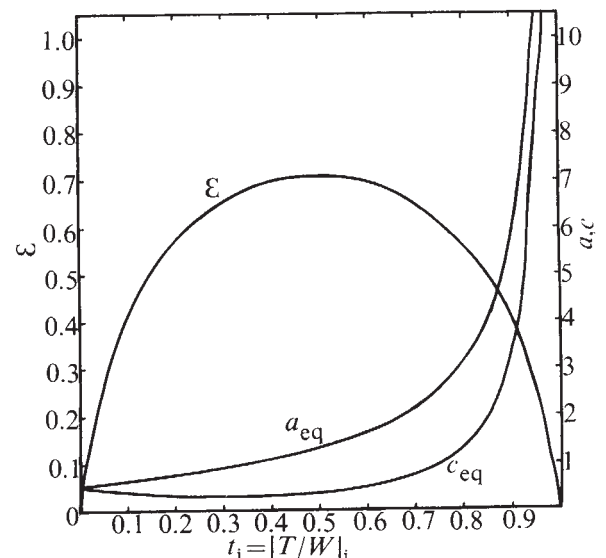
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Why there are no elliptical galaxies flatter than E7

It has long been known¹⁻³ that the flattest elliptical galaxies observed are of type E7, corresponding to an axial ratio of 3.33:1 whereas SO and spiral galaxies are seen with axial ratios up to 20:1. We show, using Maclaurin spheroid models, that an initially spherical protogalaxy, composed entirely of stars, will always relax after a dissipationless collapse to an equilibrium configuration with $\varepsilon \equiv 1 - (c/a) \leq 0.70925$, where a, c are the semi-major, semi-minor axes. This happens regardless of original angular momentum of the galaxy, and is in remarkable agreement with the maximum observed flattening of elliptical galaxies.

According to the standard cosmological picture, protogalaxies begin as small density perturbations at recombination, expand to a maximum radius and recollapse, reaching the maximum collapse point at $t = T_c$. By $t = T_c/2$, the protogalaxy has acquired its angular momentum by tidal interaction⁴ or turbulence⁵. At this time, we assume the protogalaxy to be a uniformly dense sphere of mass M , of initial radius a_i

Fig. 1 Ellipticity ε , semi-major and semi-minor axes (a, c) of the equilibrium elliptical galaxy as a function of $t_i = |T_{rot}/W|$. The curve $\varepsilon(t_i)$ is symmetrical with respect to $t_i = 0.5$.



rotating uniformly with angular velocity Ω_i . There are initially no random velocities. To characterise the angular momentum of the protogalaxy, we introduce the convenient parameter t_i as the ratio of the rotational kinetic energy T_{rot} of the sphere to its potential energy W :

$$t_i = \left| \frac{T_{\text{rot}}}{W} \right| = \frac{\Omega_i^2 a_i^3}{3GM} \quad (1)$$

The initial binding energy of the protogalaxy is:

$$E_i = W_i (1 - t_i) = -\frac{3}{5} \frac{GM^2}{a_i} (1 - t_i) \quad (2)$$

If star formation occurs early and is completed by $t = T_c/2$, the protogalaxy undergoes a dissipationless collapse¹³, relaxes violently⁶ and settles into an equilibrium configuration by $t = 3T_c/2$ (ref. 7). Violent relaxation tends to isotropise random velocities, as suggested by an N -body calculation including infall and tidal effects⁸, and by fits to the light distributions of elliptical galaxies using King's⁹ models which have an isotropic distribution of random stellar velocities. We adopt as the simplest model for the equilibrium galaxy a Maclaurin spheroid in virial equilibrium with uniform rotation and isotropic pressure. Although real elliptical galaxies have dense cores and exhibit differential rotation the ellipticities predicted by numerical models of elliptical galaxies and Maclaurin spheroid models are in reasonable agreement¹³. The energy of the Maclaurin spheroid is

$$E_{\text{eq}} = \frac{W_{\text{eq}}}{2} = -\frac{3}{10} \frac{GM^2}{a_{\text{eq}}} \frac{\sin^{-1}e}{e} \quad (3)$$

where eq denotes 'equilibrium' and

$$e = \left[1 - \left(\frac{c}{a} \right)^2 \right]^{\frac{1}{2}}$$

Since there is no dissipation, $E_i = E_{\text{eq}}$ or

$$2(1 - t_i) = \frac{\sin^{-1}e}{e} \frac{a_i}{a_{\text{eq}}} \quad (4)$$

Angular momentum conservation gives

$$(T_{\text{rot}})_{\text{eq}} = \left[\frac{a_i}{a_{\text{eq}}} \right]^2 (T_{\text{rot}})_i = \frac{3}{5} \frac{GM^2}{a_i} t_i \left[\frac{a_i}{a_{\text{eq}}} \right]^2 \quad (5)$$

The rotational kinetic energy of a pressure supported equilibrium Maclaurin spheroid is¹⁰

$$[T_{\text{rot}}]_{\text{eq}} = \frac{3GM^2}{10a_{\text{eq}}e^3} [(3 - 2e^2) \sin^{-1}e - 3e(1 - e^2)]^{\frac{1}{2}} \quad (6)$$

Combining equations (4) to (6):

$$t_i(1 - t_i) = \frac{1}{4} \frac{\sin^{-1}e}{e^4} [(3 - 2e^2) \sin^{-1}e - 3e(1 - e^2)]^{\frac{1}{2}} \quad (7)$$

which for a given t_i , can be solved for e ; a_{eq} and c_{eq} are then found using equation (3) and (4). The results are shown in Fig. 1. Note that equation (7) is symmetrical about $t_i = 0.5$ which corresponds to an ellipticity of 0.70925. For $t_i < 0.5$ (probably in the majority of cases), the galaxy gets flatter on adding angular momentum, but past $t_i = 0.5$ adding angular momentum makes the equilibrium galaxy rounder. Given our model, no elliptical galaxy can be flatter than an E7, no matter how fast it rotates initially. This surprising fact is due to the equilibrium Maclaurin spheroid blossoming to infinite size with only a finite amount of angular momentum. At $t_i = 1$,

the galaxy has only $\sqrt{2}$ times the angular momentum of one at $t_i = 0.5$ (initially in virial equilibrium), but because the total kinetic energy has increased to equal the potential energy, the binding energy becomes 0, and a_{eq} and c_{eq} become infinite. For $t_i > 1$, the galaxy becomes unbound. Another symmetry about $t_i = 0.5$ is found in numerical experiments by Bouvier and Janin¹¹ who found that spherical systems with t_i (random) = 0.25 and t_i (random) = 0.75 have comparable amounts of violent relaxation. The system with the least violent relaxation is the E7 galaxy with $t_i = 0.5$.

Any initial random velocities, or any net expansion or contraction of the initial spherical protogalaxy, can only decrease the ellipticities, since all these effects decrease the binding energy without affecting the angular momentum; thus the upper limit of $\epsilon = 0.70925$ is left intact. Maclaurin spheroids become dynamically unstable at $|T_{\text{rot}}/W| = 0.273$ ($e = 0.95289$ or $\epsilon = 0.69668$ (ref. 11)). This is close enough to our maximum value of 0.70925 not to affect our conclusions. Real stellar systems with $|T_{\text{rot}}/W| \gtrsim 0.14$ ($e > 0.81267$ or $\epsilon > 0.41727$) may, however, be unstable to bar instabilities¹². Phase mixing may smear the bar out in time so as to leave a more centrally condensed axisymmetric galaxy with $|T_{\text{rot}}/W| \sim 0.14$. It is not clear, however, that the final ellipticity is changed greatly by this process.

Our result depends on the assumption of an initially spherical state and of conservation of energy and angular momentum. The final Maclaurin spheroid can be as flat as one wants if energy is dissipated—which we believe is the mechanism for the formation of a disk spiral galaxy¹³—or if the initial state is non-spherical. To investigate this, we write:

$$E_{\text{eq}} = \alpha E_i \quad (8)$$

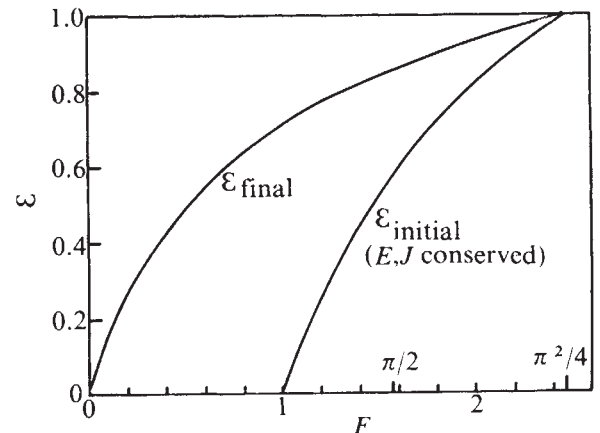
$$[T_{\text{rot}}]_{\text{eq}} = \beta \left(\frac{a_i}{a_{\text{eq}}} \right)^2 [T_{\text{rot}}]_i \quad (9)$$

$$\text{and} \quad W_i = -\frac{3}{5} \frac{GM^2}{a_i} \frac{\sin^{-1}e_i}{e_i} = -\frac{3}{5} \frac{GM^2}{a_i} \gamma \quad (10)$$

This has the consequence of multiplying the left hand side of equation (7) by $\alpha\beta\gamma$. Let $F = 4\alpha\beta\gamma t_i(1 - t_i)$. Figure 2 shows ϵ_{final} as a function of F . $\epsilon_{\text{initial}}$ as a function of F is also shown in the case when the energy E and the angular momentum J are conserved. There are several points to be made:

- (1) For the final state to be a flat disk $F = \pi^2/4$, so if J is conserved and the initial state is spherical then the dissipation must be large enough so that $E_f/E_i \geq \pi^2/4$.
- (2) In the tidal interaction picture, the angular momentum

Fig. 2 Ellipticity ϵ_{final} of the equilibrium elliptical galaxy as a function of $F = 4\alpha\beta\gamma t_i(1 - t_i)$. The initial ellipticity ϵ_i is also given as a function of F when energy and angular momentum are conserved.



is gained over a time scale $\sim T_c/2$, so for $t_i \gtrsim 0.5$ the original spherical shape may become somewhat flattened in the process of gaining angular momentum, which would have the consequence of increasing the maximum ellipticity ϵ_m . For example, for $\epsilon_i = 0.5$, Fig. 2 shows that for a dissipationless collapse, $\epsilon_{\max} \sim 0.83$ ($F = 1.48$). The limiting case is again given by $F = \pi^2/4$ when $\epsilon_i = \epsilon_m = 1$.

In conclusion, with a roughly spherical shape initially and a dissipationless collapse conserving angular momentum, we expect no equilibrium elliptical galaxies flatter than E7 regardless of the original angular momentum. The remarkable agreement of this with the maximum observed ellipticity would seem to argue that this very simple picture may be a close approximation to reality.

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TRINH X. THUAN

*Institute of Astronomy,
University of Cambridge, and
Hale Observatories,
California Institute of Technology,
Carnegie Institution of Washington,
Pasadena, California 91109*

J. RICHARD GOTT, III

*Institute of Astronomy,
University of Cambridge,
Cambridge, UK*

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Galactic dust lanes and lunar soil

It has been proposed by McCrea¹, Shapley² and Hoyle and Lyttleton³ that passages of the Solar System through interstellar clouds have appreciable effects on the Earth. McCrea argues that the recurrence of ice epochs⁴ every ~ 250 Myr coincides with the passage of the Solar System through a galactic spiral arm approximately every 10^8 yr. We report here studies on the character and grain-size distribution of texturally-mature lunar soils which support these views. The evidence shows the flux of micrometeoroids ($\lesssim 10^{-6}$ g) at the lunar surface has remained in quasi-equilibrium near the present-day value over the past 2×10^9 yr, but that significant increases have occurred. Three near-cyclical enhancements are superimposed on the drill core stratigraphy, with separations of $\sim 10^8$ yr. The magnitudes, durations, and periodicity of the flux increases suggest their origin may be the passage of the Solar System through dust lanes in the galactic spiral arms.

It has been noted⁵ that a segment of the cumulative grain-size distribution of "texturally-mature"⁶ lunar soils closely parallels the present meteoroid flux distribution in the soil grain-size range 44–177 μ m, at the maximum concentration of impact-derived constructional glass particles, or "agglutinates"^{7,8}. We believe that this segment of the soil distribution provides information on the meteoroid flux distribution that produced it.

Most of the kinetic energy in the present meteoroid flux⁹ is in particles of masses $< 10^{-6}$ g which strike the lunar soil at a

velocity of ~ 20 km s⁻¹ and generate enough heat to fuse a volume of soil. Some melt is lost as spray during crater excavation, but morphological information in the form of ring- (Fig. 1) or bowl-shaped agglutinates¹⁰ suggests that only one large agglutinate particle is formed by any one micrometeoroid impact. Thus the parallelism between grain size and meteoroid flux is understandable: one simply mirrors the other. Experimental data on the ratio of mass of the melt to mass of the meteoroid at 20 km s⁻¹ is lacking, but theory⁹ suggests that an agglutinate represents ~ 5 times the mass of the impacting micrometeoroid at 20 km s⁻¹. Using mean agglutinate diameters and a particle density of 3.1 g cm⁻³ the parallel segment of the soil curve converts to a micrometeoroid mass range of 0.08×10^{-6} – 2.0×10^{-6} g.

A meteoroid distribution is $N = am^\beta$, where N is the total number of particles larger than mass m , α is the intercept and β is the slope of a plot of log flux against log mass for masses larger than $\sim 10^{-7}$ g (refs 9 and 11). We convert the bulk grain-size distribution for the 45–177- μ m range from weight % retained in sieves to particle number equivalents assuming spherical particles. The numbers of particles in five size intervals are normalised to the total number, and we construct a cumulative particle number frequency distribution. Using equivalent meteoroid masses and cumulative particle numbers in log-log form, a linear least squares fit gives β . But α (which gives total flux) cannot be found without knowing the meteoroid exposure time of the soil. We derived β for 61 samples—12 surface soils and 49 from the Apollo 15 deep drill core. In all cases the linear fit explains $> 97\%$ of the variance, thus demonstrating a strong linear dependence between log N and log m .

Soils from the lunar surface should give β estimates near the modern flux value derived from other methods, thus offering an independent test of the model. The present meteoroid flux in our mass range has $\beta = -1.213$ (ref. 9). Estimates of β for the surface samples range from -1.266 to -1.142 with a mean of -1.213 ± 0.039 . A one-tailed t test indicates no significant difference between the modern β value and our estimates from

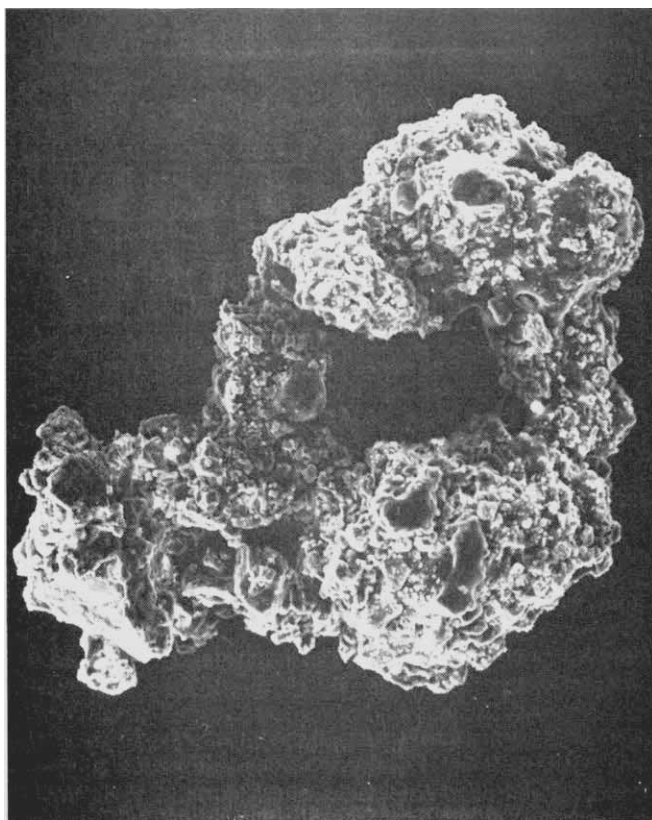


Fig. 1 A ring-shaped agglutinate from the lunar soil. The agglutinate is approximately 180 μ m in diameter.

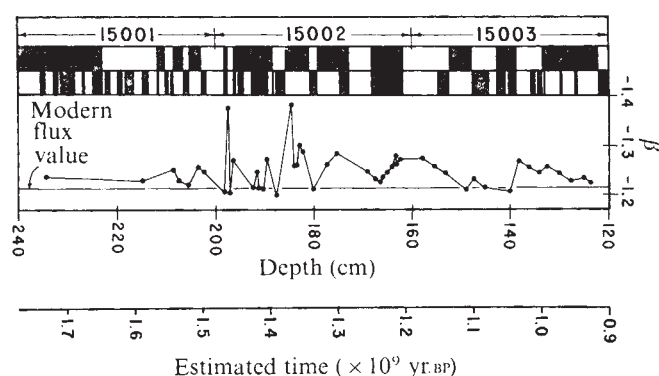


Fig. 2 Estimates of β as a function of depth in the lunar soil. Estimates of β seldom exceed the modern flux value and are not correlated with the soil stratigraphy. The top half of the stratigraphic column shows major units only—the bottom half shows all units identified.

the surface soils ($t_{0.05} = 2.201 > 0.043$, d.f. = 11). That is, the model works.

Large meteoroids produce ejecta blankets of sufficient thickness and extent to survive as layers. This creates three interpretation problems: (1) inverted stratigraphy may appear in the record¹²; (2) old agglutinates may survive excavation from an earlier layer; and (3) the soil accumulation rate is nonlinear^{8,13}.

First, inverted stratigraphic sequences of small thickness will not destroy regular trends in the data, but will increase deviations about the trend. The range in β values is not affected. Second Lindsay⁵ investigated agglutinate survival under reworking and found most pre-existing agglutinates are destroyed in excavation by layer-forming events (that is, crushed to sizes $\lesssim 15 \mu\text{m}$, below our range). Third, soil accumulation rates are nonlinear^{8,13}, but modelling the growth requires assumptions of the meteoroid flux history, so it is best to model linear accumulation. The age of the substrate¹⁴ and total soil thickness at the Apollo 15 site¹⁵ give an average accumulation rate of $1.35 \times 10^{-7} \text{ cm yr}^{-1}$. Nonlinear soil models¹³ fortunately give similar rates for this time period. Since our sampling interval is 0.5 cm our time resolution is $\sim 4 \times 10^6 \text{ yr}$.

The core samples cover a depth of 120–240 cm in known stratigraphy (Fig. 2). Estimates of β for these samples range from -1.199 to -1.381 with a mean of -1.248 ± 0.036 . The data do not show a monotonically decreasing flux distribution but suggest a quasi-steady-state background level near the modern flux value, with enhancements superimposed. Below 180 cm the deviations are a series of disordered 'spikes'; above 180 cm three regular deviations each extend over 15–20 cm of the record. Each cycle crosses several stratigraphic units, indicating that the β variations have extra-lunar, time-dependent sources, rather than being determined by independent depositional processes operating on individual stratigraphic units.

Micrometeoroid experiments on the Pioneer 8, 9, 10 and 11 spacecraft^{18,19} show the asteroid belt is not a small particle source. Unless the particle populations and dynamics in the Solar System were quite different 1–2 eons ago, we suggest that the flux enhancements are not redistributions of matter by asteroidal collisions. We propose the enhancements record passages of the Solar System through dense interstellar clouds, plus active cometary episodes. These two sources may be manifestations of the same event: namely the passage of the Solar System through compression lanes in the arms of the Galaxy¹ and the concomitant generation of comets and their subsequent mass loss to the Solar System²⁰.

Lyttleton^{20,22,25} suggests that comets are "new" members of the Solar System formed by the interaction of the Sun with interstellar clouds. The Sun encounters dense clouds ($\sim 10^{-19} \text{ g cm}^{-3}$) in "compression lanes" of interstellar matter in the galactic spiral arms²⁰. Mass loss from long-period comets so

formed could enhance the small particle flux for 10^7 – 10^8 yr (ref. 17).

Hartung and Störzer¹⁶ suggest the modern small particle flux is increasing due to activity by Comet Encke. A micro-meteoroid detector on the Heos-2 spacecraft²¹ shows that Comet Kohoutek deposits small particles into the inner Solar System. Lyttleton²⁰ suggests that particles $\lesssim 10^{-6} \text{ g}$ are lost from comets due to the solar wind. Since comets have masses of 10^{17} – 10^{20} g and lose $\gtrsim 10^{-4}$ of this in each solar encounter^{20,22}, one comet could increase the particulate material density within the orbit of Jupiter²⁰ by $\sim 3 \times 10^{-24} \text{ g cm}^{-3}$ if the debris is in the ecliptic. For matter collected by the Moon at 20 km s^{-1} , the surface flux would increase by $\sim 10^{-10} \text{ g cm}^{-2} \text{ yr}^{-1}$ above the present level of 1.2×10^{-9} – $4 \times 10^{-9} \text{ cm}^{-2} \text{ yr}^{-1}$ in small particles^{9,11,23,24}. Further flux enhancements are possible for cometary periods which are short compared with the Solar System debris retention time¹⁷. Such enhancements could explain brief flux excursions. The 'peak' at $\sim 200 \text{ cm}$ corresponds to a small particle flux increase of ~ 15 times the present value.

The speed²⁶ of the Sun through compression lanes is 5 – 24 km s^{-1} , so cloud densities as above give enhancements $5 \times 10^{-6} \text{ g cm}^{-2} \text{ yr}^{-1}$. Although interstellar clouds have variable constitution, "typical clouds" contain $\sim 1\%$ grains by mass^{27,28}. For an encounter speed of 20 km s^{-1} , these particles striking the Moon would increase the small particle flux by $\sim 10^{-8} \text{ g cm}^{-2} \text{ yr}^{-1}$. So such encounters are numerically capable of producing the flux changes deduced from lunar soil parameters.

From 120 to 180 cm in the core tube the β variations are smooth, cyclical deviations from the present-day value. Although it is difficult to correlate depth with age exactly, the three cycles have periodicities of 1×10^8 – $2 \times 10^8 \text{ yr}$ and comparable durations. On this basis, the cycles cover an age 0.9×10^8 – $1.5 \times 10^8 \text{ yr}$ (all Precambrian), so unfortunately our data cannot be compared with known glacial epochs. McCrea¹ points out that the Solar System crosses a spiral arm every 10^8 year or so, taking $\sim 10^6 \text{ yr}$ to cross the compression lane and spending a total of $\sim 10^7 \text{ yr}$ in the arm. This agrees well with our estimates of β cycles in the soil, except for their longer durations ($\sim 10^8 \text{ yr}$) which may be due to mass loss to the inner Solar System by long-period comets born in such encounters.

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JOHN F. LINDSAY

Marine Science Institute,
University of Texas,
Galveston, Texas 77550

L. J. SRNKA

The Lunar Science Institute,
3303 NASA Road 1,
Houston, Texas 77058

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Jupiter's atmospheric circulation

By numerical integration of conventional meteorological equations at appropriate parameter values we have been able to reproduce most of the major characteristics of the Jovian atmosphere.

The axisymmetry and scale of the bands, the oval-shaped disturbances, the waves and the Great Red Spot are all essentially characteristics of turbulent barotropic vorticity exchanges in a rapidly rotating planetary atmosphere. They are produced by the interaction in a spherical domain of a two-dimensional (horizontal) turbulent cascade and Rossby wave propagation, a process that we shall refer to as global turbulence. This interaction occurs at a length scale $L_p = \pi(2u/\beta)^{1/2}$, where u is a measure of the zonal velocity and β is the northward gradient of the Coriolis force, that closely matches the observed size of the bands. This hypothesis has been verified by solutions obtained for a stochastically forced barotropic equation, see for example Fig. 1.

The complete Jovian thermodynamical system can be reasonably well reproduced by using a standard (that is, Phillips's) terrestrial general circulation model under Jovian parameter conditions (see Fig. 2, for example). Apart from the characteristic banded structure the solutions also reveal the existence of an intra-jet circulation or gyre in which the flow resembles that surrounding the Great Red Spot. The planet also seems to have a heat transfer (index) cycle with a 4 to 5-yr period that accounts for long term variability. Clouds are produced by vertical circulation cells induced by frictional Ekman pumping.

Our theoretical flows suggest that the Great Red Spot can be thought of as essentially the core of an intra-jet circulation or as an eddy of global turbulence (as defined above). Like the ovals,

Fig. 1 An example of simulated Jovian turbulence. Sphere contains stream function contours with negative values shaded by 1/4 of grid points. Profile of longitudinally averaged zonal flow has a scale of 100 m s^{-1} in right-hand side diagram. State is transient and early in atmospheric evolution to final form. Although more orderly than final state it illustrates basic elements more clearly. A cine film of this solution is available on request.

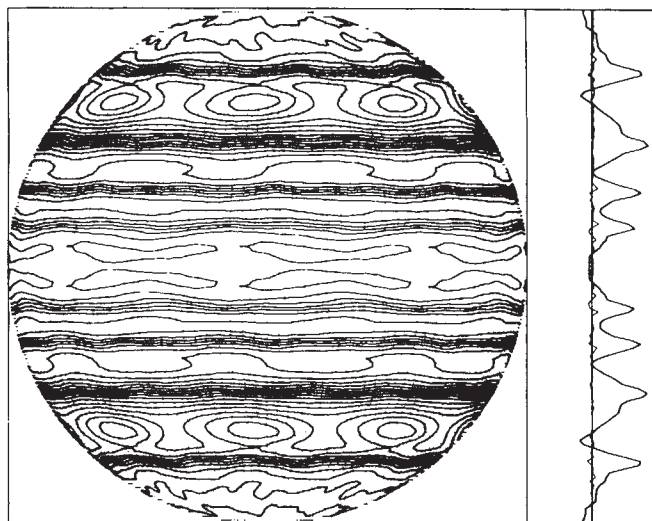


Fig. 2 The global circulation as given by a terrestrial general circulation model integrated in the Jovian parameter range. Calculations were made over a limited sector and repeated for global display. Model is not valid at the equator.

the Great Red Spot plays an important role in the energy cascade that maintains the multiple zonal currents. The persistence of the Great Red Spot is due to the fact that (1) energy cascades toward larger scales in two-dimensional turbulence and (2) under the appropriate conditions large intra-jet circulations such as that forming the Great Red Spot are an integral part of this type of multiple-jet global circulation.

Thus Jupiter's atmosphere seems to have the same dynamical ingredients as the terrestrial atmosphere and ocean. The processes occur on different scales, however, and act in different proportions. As in the terrestrial and Martian systems, baroclinic instability again seems to be the primary energy conversion process. A complete description of the meteorology of Jupiter and Saturn has been submitted for publication elsewhere.

GARETH P. WILLIAMS

*Geophysical Fluid Dynamics Laboratory,
National Oceanic and Atmospheric Administration,
Princeton University,
PO Box 308
Princeton, New Jersey 08540*

Received May 22; accepted September 11, 1975.

Noble gases in an Hawaiian xenolith

THE noble gas record in meteorites and lunar samples has been the subject of many investigations aimed at determining their age, the history of their exposure to cosmic rays and to the solar wind, and the early chronology of events in the Solar System (see review in ref. 1). Information on the latter is contained primarily in the isotopes of xenon, where the decay products of extinct ^{129}I and ^{244}Pu provide a record of the synthesis of elements and the early history of planetary solids (see review in ref. 2). The occurrence of radiogenic xenon in CO_2 well gas from Harding County, New Mexico is the only clear evidence that extinct radioactivities were present in the early history of the Earth^{3,4}, but the suggestion that this radiogenic xenon had been brought near the Earth's surface in hot magmas was not confirmed by recent analyses of xenon in lava rock from this region⁵.

The present investigation of noble gases in a volcanic xenolith containing high-purity inclusions of liquid CO_2 was undertaken to see if radiogenic xenon might be associated with

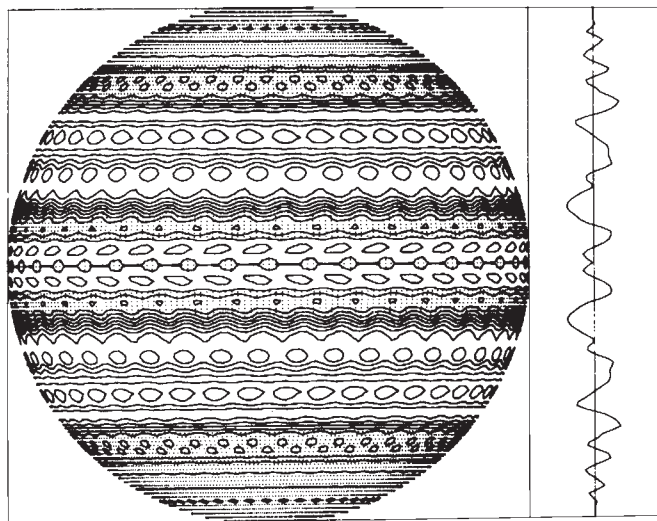


Table 1 Concentrations of noble gases in xenolith

Sample	I	I	Blank I	II	II	Blank II
Weight (g)	21.307	21.307	—	15.136	15.136g	—
Temperature (°C)	600	1,700	1,700	600	1,700	1,700
Concentrations (cm ³ g ⁻¹ at STP)						
⁴ He × 10 ⁸	1.1	32	1.5	1.2	39	1.8
²² Ne × 10 ¹¹	0.52	8.9	2.4	0.56	6.7	1.3
³⁶ Ar × 10 ¹⁰	0.37	4.2	0.31	0.16	3.7	1.1
⁴⁰ Ar × 10 ⁷	0.11	13	0.09	0.47	16	0.31
⁸⁴ Kr × 10 ¹²	0.32	5.8	0.32	0.12	5.6	0.35
¹³⁰ Xe × 10 ¹⁴	0.55	12	0.46	0.34	13	0.40

mantle-derived CO₂ (ref. 6) and to see if the abundance pattern of noble gases within the Earth is represented by the noble gases trapped in glassy margins of certain deep-sea basalts^{7,8}. Two relatively large samples were taken from the interior of a large peridotite xenolith (≈0.5 kg) which had been brought to the surface by the 1801 Kaupulehu flow of the Hualalai volcano, Hawaii. The xenolith is composed of large olivine crystals (≈1–10 mm), which have been shown by density measurements and X-ray analysis to be rich in magnesium, approaching forsterite. The Hawaiian xenoliths are characterised by an abundance of inclusions of liquified CO₂, up to 3% by volume (0.5% by weight)⁹. These inclusions have been shown to contain radiogenic ⁴He and ⁴⁰Ar with a low ⁴He/⁴⁰Ar ratio as might be generated from a source of chondritic composition¹⁰.

To ensure complete autonomy of samples and to guard against the possibility of a systematic error, as might arise from 'memory' effects in the system, the two samples were analysed several months apart. Samples of other material were analysed in the interim period. The samples were mounted in the upper chamber of a water-cooled quartz extraction bottle, the pressure reduced to ≈10⁻⁸ mmHg, and the system blank measured by analysing gases generated in heating the new, empty molybdenum crucible to the highest temperature (≈1,700 °C) to be used in extracting gases from the sample. When the system blank had reached an acceptable level, a system blank for the low extraction temperature (600 °C) was determined, and then the sample was dropped into the crucible for extraction of gases. The gases were collected and analysed at the two extraction temperatures, first at ≈600 °C and then at ≈1,700 °C, at which the sample completely melted. The procedures used in our laboratory for calibration of the mass spectrometer and for extraction, clean-up, and analysis of noble gases have been described previously¹¹.

The concentrations of noble gases found in these samples are presented in Table 1. The gas concentrations were measured by the 'peak height' method, and are estimated to have an error of ±20%, except that ⁴He may have a systematic error due to leakage of helium through our glass vacuum system or through our glass air standards. To compare the abundance pattern of non-radiogenic noble gases in the xenolith with that in other terrestrial materials, we define a fraction factor, f^m , relative to ³⁶Ar,

$$f^m = ({}^m\text{X}/{}^{36}\text{Ar})_{\text{sample}} / ({}^m\text{X}/{}^{36}\text{Ar})_{\text{air}} \quad (1)$$

where X represents a non-radiogenic noble gas isotope of mass number, m . For atmospheric noble gases¹² $f^m \equiv 1.00$ at all values of m , and, for all samples, $f^{36} \equiv 1.00$.

Figure 1 compares the values of f^m for noble gases in the xenolith with values of f^m for noble gases in Fig Tree Shale¹³ and in an enstatite melt which equilibrated with atmospheric noble gases¹⁴. The relative abundances of noble gases in Fig Tree Shale show the effect of preferential adsorption of the heavier noble gases^{15,16}, and the gases in the enstatite melt show that a solution of noble gases in melts in equilibrium discriminates against incorporation of the larger atoms. The abundance pattern of Ne, Ar and Kr in the xenolith is very close to that predicted by Kirsten¹⁴ for an enstatite melt which

has equilibrated with a gas reservoir having the relative abundances of Ne, Ar, and Kr that exist in the present atmosphere¹². For Xe the trend is reversed, however, and the xenolith has approximately 10 times more Xe than expected in a melt which has equilibrated with atmospheric noble gases. An apparent excess of Xe in the xenolith can be explained in terms of the selective depletion of atmospheric Xe by adsorption on shales^{13,15,16}. In view of the fact that Hawaiian basalt magma and xenoliths may come from depths as great as 60 km (ref. 6), the abundance pattern of noble gases in the xenolith (Fig. 1) suggests that the relative abundances of Ne, Ar, and Kr in the atmosphere may be a fair representation of noble gases which equilibrated with the mantle, but the Xe/Ar ratio in the mantle may be about an order of magnitude higher than the Xe/Ar ratio in the atmosphere. Thus, the abundance pattern for the total terrestrial inventory of noble gases seems to be very similar to that in typical chondrites, as has been suggested earlier from studies of noble gases on shales^{13,15,16}.

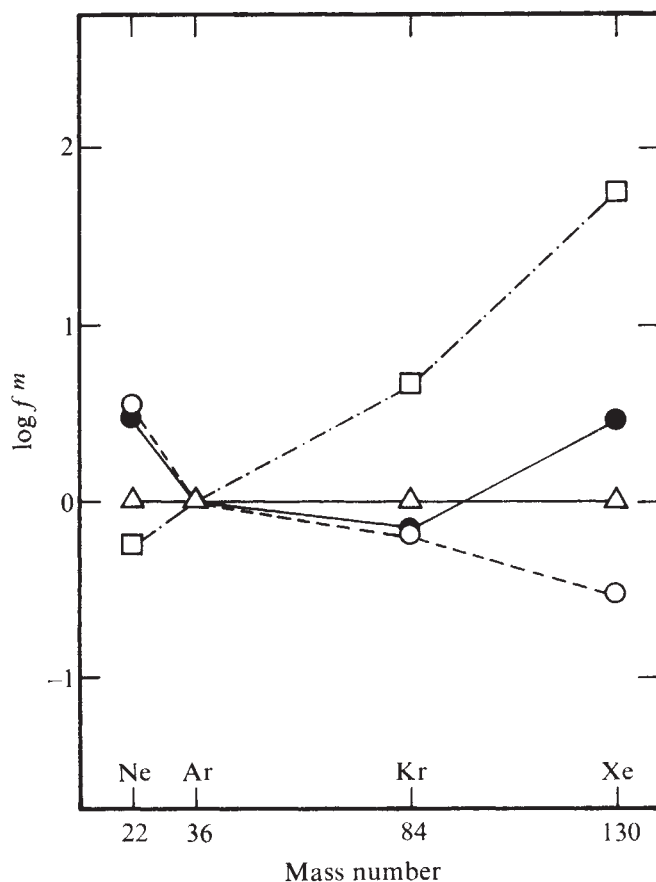


Fig. 1 A comparison of the noble gas abundance pattern in xenolith (●) with that in air (△)¹², in Fig Tree Shale¹³ (□), and in an enstatite melt¹⁴ (○), which has equilibrated with atmospheric noble gases.

The isotopic compositions of the noble gases released from the xenolith at 1,700 °C are given in Table 2, together with the isotopic composition of atmospheric noble gases. Xenon displays a clear excess of ^{129}Xe and a small excess of ^{136}Xe which is too low for a determination of the fission yields. The isotopic composition of the non-radiogenic xenon seems to be atmospheric, and the isotopic compositions of the other noble gases reveal no anomalies, except for radiogenic ^{40}Ar . The relatively high concentrations of ^{40}Ar interfered with measurements of other isotopes of argon and neon. The high beam intensity of $^{40}\text{Ar}^+$ ions caused a rapid build-up of 'memory' peaks at ^{36}Ar and ^{38}Ar , and $^{40}\text{Ar}^{2+}$ ions interfered with the ^{20}Ne measurement. For these reasons the isotopic composition of neon is not reported in Table 2, and only upper limits are given for the $^{38}\text{Ar}/^{36}\text{Ar}$ ratio.

The 4.5% enrichment of ^{129}Xe is greater than that observed in any well gas⁴ except in the CO_2 gas from Harding County, New Mexico, and the ^{129}Xe enrichment here is almost twice as large as that first observed³ in the CO_2 well gas. It should be stressed again that there were no indications during the analyses of memory or contamination at mass number 129, and we are confident that the ^{129}Xe anomaly is real. Possible origins for excess ^{129}Xe in

use of the noble gases in the xenolith to decipher the abundance pattern of noble gases within the Earth.

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E. W. HENNECKE
O. K. MANUEL

Nuclear Division,
Department of Chemistry,
University of Missouri,
Rolla, Missouri 65401

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Table 2 Isotopic composition of noble gases in xenolith			
Sample	I	II	Atmosphere
Weight (g)	21.307	15.136	—
Temperature (°C)	1,700	1,700	—
$^{36}\text{Ar}/^{36}\text{Ar}$	≈ 1.00	≈ 1.00	≈ 1.00
$^{38}\text{Ar}/^{36}\text{Ar}$	≤ 0.212	≤ 0.209	0.187
$^{40}\text{Ar}/^{36}\text{Ar}$	$3,328 \pm 6$	$4,437 \pm 14$	296
$^{80}\text{Kr}/^{84}\text{Kr}$	3.96 ± 0.05	4.04 ± 0.04	3.96
$^{82}\text{Kr}/^{84}\text{Kr}$	20.3 ± 0.1	20.3 ± 0.1	20.2
$^{83}\text{Kr}/^{84}\text{Kr}$	20.2 ± 0.1	20.1 ± 0.1	20.2
$^{84}\text{Kr}/^{84}\text{Kr}$	≈ 100	≈ 100	≈ 100
$^{86}\text{Kr}/^{84}\text{Kr}$	30.6 ± 0.1	30.5 ± 0.1	30.6
$^{124}\text{Xe}/^{130}\text{Xe}$	—	2.38 ± 0.03	2.35
$^{126}\text{Xe}/^{130}\text{Xe}$	—	2.24 ± 0.03	2.21
$^{128}\text{Xe}/^{130}\text{Xe}$	—	47.6 ± 0.4	47.0
$^{129}\text{Xe}/^{130}\text{Xe}$	673 ± 3	677 ± 2	648
$^{130}\text{Xe}/^{130}\text{Xe}$	≈ 100	≈ 100	≈ 100
$^{131}\text{Xe}/^{130}\text{Xe}$	518 ± 2	518 ± 2	519
$^{132}\text{Xe}/^{130}\text{Xe}$	658 ± 3	658 ± 2	659
$^{134}\text{Xe}/^{130}\text{Xe}$	258 ± 2	259 ± 2	256
$^{136}\text{Xe}/^{130}\text{Xe}$	220 ± 2	221 ± 1	217

The isotopic compositions of atmospheric Ar, Kr and Xe are from refs 17, 18 and 19, respectively.

CO_2 well gas have been considered elsewhere³, and the conclusion that this represents the decay product of extinct ^{129}I applies equally well to the xenolith sample. The xenolith sample thus represents the first terrestrial rock in which the decay product of an extinct radioactivity has been identified.

The site of the radiogenic ^{129}Xe in the xenolith was not determined, but the report of radiogenic ^4He and ^{40}Ar in the inclusions of xenoliths¹⁰ and the reports⁴ of radiogenic ^{129}Xe in CO_2 well gas suggest that we may be seeing 'orphan' ^{129}Xe , that is, radiogenic ^{129}Xe from an external reservoir rather than radiogenic ^{129}Xe produced within the xenolith by the decay of ^{129}I . Green⁶ has suggested the possibility of a CO_2 -charged asthenosphere as a source for the CO_2 -rich fluid inclusions in Hawaiian xenoliths. His model implies that the solid Earth is effectively capped against diffusive outgassing by the CO_2 asthenosphere, a result which may account for the terrestrial association of radiogenic ^{129}Xe with CO_2 . The presence of this decay product of ^{129}I ($t_{1/2} = 17 \times 10^6$ yr) in the xenolith is further evidence that the formation of the Earth did not appreciably postdate the formation of meteorites. It also indicates that the xenolith contained juvenile noble gases from the interior of the Earth, and thus lends credence to our

Bottom sliding of a glacier measured from the surface

WE have measured the sliding motion at the bottom of a glacier from the surface of the ice. Fleming Glacier in the Antarctic Peninsula is a fast flowing polar glacier, more than 1,000 m thick, feeding the Wordie Ice Shelf. Observations indicate that up to a third of its surface velocity is due to the bottom layers of ice sliding over the bed. Previous measurements of the bottom sliding of glaciers have been confined to tunnels or boreholes that have reached bed-rock^{1,2}; sliding velocities have also been calculated indirectly by using assumptions about the internal deformation of ice³. Results obtained by our method can help to test theories of basal sliding⁴ and should give insight into the mechanism and consequences of surges in glaciers and ice sheets⁵.

The surface velocity can be considered to consist of two components, one representing deformation of ice in the body of the glacier and the other representing basal slip. Conventional optical survey using fixed rock as a reference was used to give the sum of both components of the surface velocity. A radio-echo technique⁶ gave the surface velocity relative to a layer that reflected electromagnetic waves—evidently from a level close to the base of the ice. Since this velocity differed from the survey velocity, it was inferred that the reflecting layer was embedded in the ice. The radio-echo method thus measured only the deformation component of the surface velocity, and the difference between the survey and radio-echo velocities represented the bottom sliding velocity.

Walford⁷ tried the radio-echo technique on Fleming Glacier and concluded that the radio-echo velocity agreed, within his limits of error, with the surface velocity determined by optical survey. We repeated the experiment, at a site within 1 km of Walford's site, in January 1974, but over a longer time to increase accuracy. This time there was a significant difference between the survey and radio-echo velocities⁸. In December 1974, therefore, we investigated

Table 1 Observed surface velocities on Fleming Glacier

	Date 1974	1		2		3	
		m yr ⁻¹	Direction	m yr ⁻¹	Direction	m yr ⁻¹	Direction
Survey velocity	Jan.	201 ± 20	284 ± 5°				
	Dec.	201 ± 4	283 ± 5°	175 ± 4	272 ± 5°	146 ± 4	277 ± 5°
Radio echo velocity	Jan.	138 ± 5	287 ± 1°				
	Dec.	142 ± 11	277 ± 1°	113 ± 7	272 ± 1°	139 ± 7	271 ± 1°
Sliding velocity	Jan.	62 ± 25	284 ± 5°				
	Dec.	59 ± 15	283 ± 5°	64 ± 11	272 ± 5°	7 ± 11	277 ± 5°

Relative velocity between sites 1 and 2 in December 1974 was 29 ± 4 m yr⁻¹ at $272 \pm 1^\circ$. The direction of the sliding velocity has been taken to be that of the survey velocity.

this same site and also two other sites on a flowline upstream from it, to resolve the apparent discrepancy. Figure 1 shows the radio-echo ice thickness profile through the sites occupied in December 1974, and Table 1 gives a summary of the results from the two seasons' work.

The optical survey was carried out by a method of resection. Six permanent reference objects on surrounding mountains were intersected from each end of a baseline 417.45 m long in January 1974, and 5,552.66 m long in December 1974. Subsequent resection of these points gave the movement of each site (J. L. W. Walton, personal communication). A period of about 16 d was necessary between resections to obtain the required accuracy. The change in distance between sites 1 and 2 was measured by a pair of MRA-3 tellurometers, which over a period of 14 d gave the relative velocity between the sites. This agreed with the difference between observed surface velocities at the two sites.

We used a modified Scott Polar Research Institute Mark IV radio-echo sounder to record the bottom-echo fading pattern only. It operated at 35 MHz in January 1974 and at 60 MHz in December 1974. Fading patterns over an area 11×2.5 m were generated by moving the aerial in a series of parallel lines along a track 25 cm above the surface of the snow. The patterns were calibrated with marks representing distance moved from a reference stake fixed in the surface of the glacier. The displacement of successive patterns relative to the stake over a period of days, was measured by comparing the position of distinctive features in the pattern with the distance marks. Values from parallel runs were averaged to give the velocity component along the track; a maximum error of 0.5%, resulting from the difference in angle between the survey velocity and the

alignment of the radio-echo track, can be neglected. Radio-echo velocities were obtained from fading patterns taken over periods of up to 10 d.

Table 1 shows that there is excellent agreement between the January and December results at site 1. Walford's radio-echo velocity at his site (close to our site 1) of 139 ± 11 m yr⁻¹ along $260 \pm 10^\circ$ also agrees with ours, indicating that differences between survey and radio-echo velocities are significant, and due to sliding of the glacier over its bed. Results from site 2 show a similar behaviour, but site 3 seems to have no sliding component. We are not yet in a position to determine whether there really was no sliding at site 3. If sliding did occur but the radio-echo reflecting surface was the actual glacier bed, rather than a layer embedded in the ice near the base, this would account for the anomaly. If the reflecting surface was the true glacier bed it is to be expected that the radio-echo velocity would be the same as the survey velocity.

An ice mass can only slide over its bed if the temperature of the basal ice is at its pressure melting point. Using measured values of the ice temperature at a depth of 10 m (-12.6°C), the observed annual accumulation of snow (100 g cm^{-2}) at site 1 and the surface velocity transformed to an equivalent basal heat flux⁹, steady-state models¹⁰ indicate that the basal ice at site 1 is probably at the melting point. This calculation cannot, however, be sufficiently precise to distinguish between basal ice temperatures at the three sites.

We noticed that the relationships between some features in the fading patterns changed with time. This may be attributed to a gradual deformation of the geometry of the reflecting layer near the base of the ice. Although the nature of the reflecting layer is unknown, it evidently reflects more electromagnetic energy than the bedrock. If the reflecting layer and the bedrock reflected nearly equal amounts of energy, then the received signal, being a combination of echoes from both reflecting surfaces, would be sensitive to relative motion between them. In this case, the fading patterns would not remain recognisable after a period of time. The reflecting layer could be either morainic material², or possibly a layer of ice at its pressure melting point.

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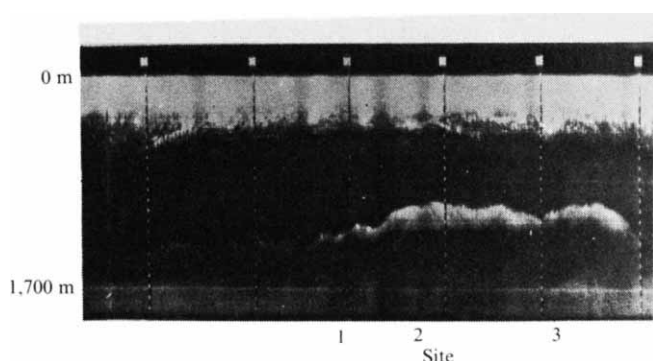
C. S. M. DOAKE

British Antarctic Survey,
Scott Polar Research Institute
Cambridge CB2 1ER, UK

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Fig. 1 Radio-echo ice thickness profile along the probable flowline through the three sites in December 1974. Site 1 was also used in January 1974. The distance between sites 1 and 2 was 5.5 km and between sites 2 and 3 was 3.0 km. The direction of ice flow is from site 3 to site 1.



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An experimental approach to lithification textures

ALTHOUGH observations on changes taking place during the compression of monominerals have been published^{1–3}, little or no information exists on the textural aspects of compression affecting granular sediments. The main reason for this lack of textural information is presumably a technical problem. Ever since the pioneering investigations conducted by Bridgman, equipment used in high pressure experiments in geology has consisted of hydraulic cylinders in combination with completely closed, thin-walled test-tube bombs compressed by the surrounding oil in the hydraulic system^{4,5}. The triaxial cell used in tectonic studies, allowing control over the three stress directions, uses massive samples jacketed to prevent the pressure medium from direct contact with the sample⁶. Triaxial studies have been performed only with massive samples, that is, already lithified rocks. No tests have yet been described involving thin sectioned samples of granular sediments subject to compression. Essentially, such experiments would reveal the mechanical aspects of the burial of natural sediment.

The pore medium used in the experiments consisted of a liquid plastic. Minerals are unlikely to dissolve in, or react chemically with, this, and the plastic acts at the same time as the embedding medium, allowing thin sectioning of the compressed sample. After mixing a small quantity of dry granular sediment (for example, quartz sand, carbonate bioclasts, calcite crystals, fragments of feldspar, garnet, topaz, beryl) with different amounts of the liquid plastic, compression took place, reaching maximum loading in minutes. Samples were compressed with a load of 7,000 kg, creating confining pressure of some 1,000 kg cm⁻². This value of 1 kbar corresponds to a depth of burial of some 4.7 km when using an average density of the sediment cover of 2.7 g cm⁻³. No means of artificial heating was used; therefore, the textural changes are caused by mechanical processes only (see also ref. 6). After hardening of the plastic, the brass ring was partially removed using a rotary cutter. After thin sectioning, samples were analysed with a polarising microscope.

Examination of numerous samples that were subjected to laboratory compaction reveals the existence of two major mechanisms responsible for the creation of lithification textures: plastic deformation and grain crushing. Two, more or less characteristic examples of the newly created lithification textures were selected (Fig. 1a and b). Rapid compression of a quantity of calcium carbonate bioclasts (clam shells, echinoid fragments, coralline algae, and encrusting algae) to 1,000 kg cm⁻² not only leads to the total disappearance of porosity (and permeability), but also causes the creation of a multitude of indentation contacts resulting from the plastic deformation of the grain contacts



Fig. 1 a, Lithification texture produced experimentally during dry compression of various carbonate bioclasts (fragments of clam shells, echinoids, coralline and encrusting algae). b, Thin sectioned natural sample (bioclast grainstone). Compare the nature of grain contacts and the absence of porosity with the experimental sample in a. c, Lithification texture after dry compression of well-rounded quartz grains. Only a few grains remained intact—highly angular fragments and cryptocrystalline quartz powder originate during brittle failure. d, Thin sectioned natural quartzite indicating the role of purely mechanical phenomena, such as grain crushing, in the creation of lithification textures in natural sediments.

As a first step in the experimental investigation of lithification textures, brass pressure cells were constructed. A massive steel piston was used to create a confining pressure in brass rings (20 mm high, with walls 2 mm thick). A first series of experiments, consisting of the compression of various dry granular sediments, revealed that a considerable degree of grain crushing took place. With granular material, shear stresses at grain contacts predominate during compression, initiating brittle failure. Compression of only a few grains in excess liquid medium, creates a hydrostatic pressure, without markedly affecting the grains. Thus, by varying the initial quantity of pore liquid mixed with the dry sample, control over pore pressure can be obtained.

(Fig. 1). Although a certain degree of grain crushing (brittle failure) takes place during the compression of the bioclasts, almost all the grains still show clear evidence of their original texture. The nature of grain contacts formed should be compared with thin-sectioned natural samples to evaluate the possible role of plastic deformation in the creation of lithification textures. As can be seen in Fig. 1b, a thin section prepared from a natural bioclast grainstone, indentation grain contacts and the total absence of porosity are also characteristics of certain natural limestones.

The second mechanism responsible for the creation of lithification textures is observed on dry compression of a well rounded quartz sand at a confining pressure of 1,000 kg cm⁻². Many of the well rounded grains break into

angular fragments and even into smaller fragments closely resembling cryptocrystalline quartz (Fig. 1c). Comparison with a natural quartzite sample (Fig. 1d) reveals once again the value of the experimental creation of lithification textures: the microfabric formed closely resembles that of the sample obtained by laboratory compression. The experiments reveal the existence of mechanisms other than the geochemical reactions postulated under the concept of "diagenesis".

In conclusion, the significance of the experimental approach in the analysis of lithification textures seems to have been established, adding an extra dimension to existing methods of geological high-pressure research.

JOHN C. DEELMAN

Laboratorium für Sedimentforschung,
6900 Heidelberg, Berlinerstrasse 19,
West Germany

Received August 8; accepted August 15, 1975.

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Stable carbon and oxygen isotope ratios of groundwaters from the Chalk and Lincolnshire Limestone

SAMPLES of groundwater have been collected from the Chalk of the London Basin and the Lincolnshire Limestone in eastern England to determine the radiocarbon and the $^{13}\text{C}/^{12}\text{C}$ ratio in the dissolved carbonate species, and the $^{18}\text{O}/^{16}\text{O}$ and D/H ratios of the water. The results will be published more fully elsewhere, but here we report preliminary findings of the $^{13}\text{C}/^{12}\text{C}$ and $^{18}\text{O}/^{16}\text{O}$ ratios and their implications.

In the London Basin the $\delta^{13}\text{C}$ changes from about -13‰ in and near the outcrop of the Chalk to values less negative than -1‰ about 8 km from the outcrop; the least negative value recorded was -0.5‰ (Fig. 1). Similarly, in the Lincolnshire Limestone, values became less negative in the direction of groundwater flow, reaching -2‰ some 15 km

from the outcrop. These low negative values are unusual. They approach those for the carbon of the London Chalk and Lincolnshire Limestone matrices of $+2.4\text{‰}$.

The radiocarbon content of carbonate species dissolved in groundwater is a function of: (1) the time that has elapsed since the carbonate was in contact with a source of modern carbon, and (2) reactions between the aquifer and the dissolved carbonate species.

The latter have an important bearing on the interpretation of the radiocarbon results. The $^{13}\text{C}/^{12}\text{C}$ ratio is essential for the evaluation of the magnitude of reactions, other than radioactive decay, which may change the ^{14}C content of the groundwater¹. The use of the $^{13}\text{C}/^{12}\text{C}$ ratio in the interpretation of ^{14}C data is based on the fact that the principal sources of the carbonate species in water give different values for the ratio. Thus, either the contribution of different species to the measured ^{14}C in the sample can be estimated, or the measurements can assist in the understanding of the groundwater chemistry. Interpretation of the carbonate chemistry is necessary to assess the correction factor that must be applied to the radiocarbon content because of dilution by non-radioactive carbon.

Although changes in the carbonate chemistry of groundwater in the London Chalk and Lincolnshire Limestone do take place in a down-gradient direction (because of ion exchange, carbonate precipitation and sulphate reduction^{2–4}), these changes alone cannot account for the very small negative values measured. The bicarbonate concentration of the water only increases from about 270 mg l^{-1} in the Chalk outcrop to about 380 mg l^{-1} in the centre of the London Basin (a distance of some 15 km). Similarly, in the Lincolnshire Limestone the increase is only from 270 mg l^{-1} in the outcrop zone to about 460 mg l^{-1} down gradient.

In view of this, the low negative values seem to be due to either isotopic exchange between the carbonate in the aquifer and bicarbonate in the water or continuous precipitation and solution of carbonate as the water flows slowly between the individual constituent particles of the limestone matrix. This view is supported by the fine-grained nature of the aquifers, particularly the Chalk, and the consequent large surface area that exists in contact with the water. Isotopic exchange is, however, a very slow process and is unlikely to have had a significant effect over the timespan during which radiocarbon dating can be applied¹. Therefore continuous precipitation and solution is favoured as the process responsible for the low negative $\delta^{13}\text{C}$ values.

On the basis of this interpretation, the radiocarbon analyses have been corrected to derive an age for the water

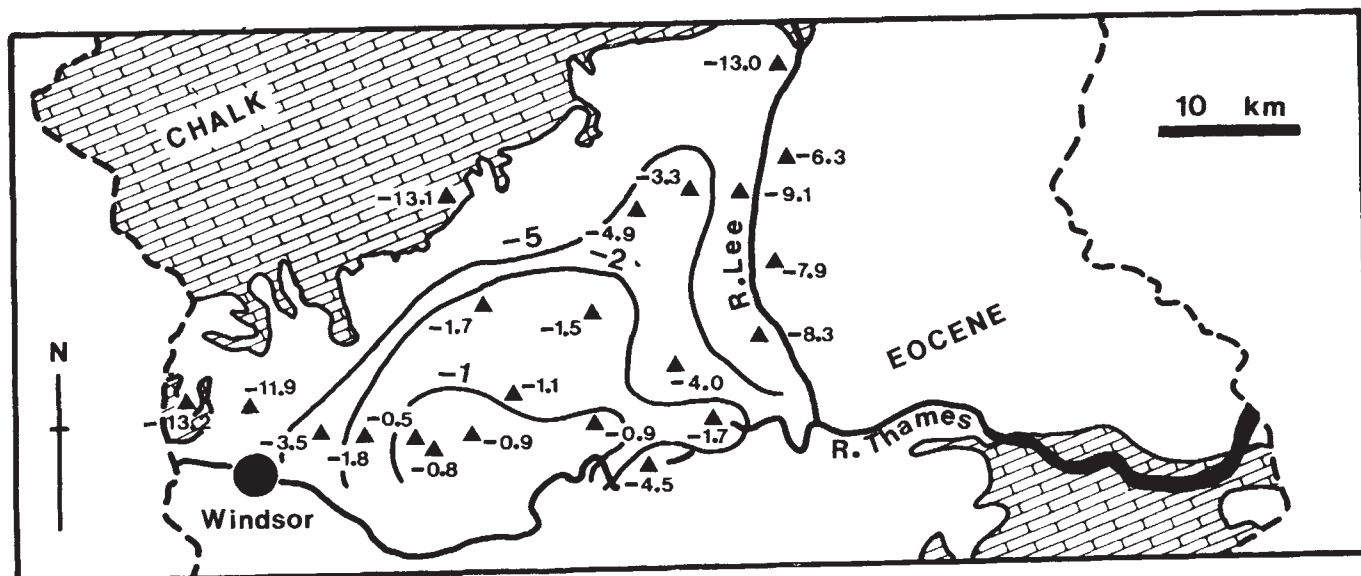


Fig. 1 $\delta^{13}\text{C}$ values (parts per 10³) for groundwater in the Chalk in part of the London Basin.

in the centre of the London Basin and in the eastern part of the Lincolnshire Limestone in excess of 25,000 yr. The correction assumes that in fine-grained carbonate aquifers, the volume of the rock greatly exceeds the carbonate precipitate, and re-solution of previously precipitated carbonate is insignificant.

An age greater than 25,000 yr implies that the water originated in rainfall during the Pleistocene. The ratios of the stable isotopes $^{18}\text{O}/^{16}\text{O}$ and D/H in this Pleistocene water are in the proportions found in present-day normal meteoric water³. The range of $\delta^{18}\text{O}$ in the Pleistocene water in the centre of the London Basin is from -7.7‰ to -7.9‰ whereas in modern water from the periphery of the Basin, the range is -7.1‰ to -7.2‰ . This suggests that either the temperatures at the time of recharge were less than one degree cooler than today³, or the weather patterns producing the recharging rainfall differed from those operating now. A possible explanation is that recharge occurred during relatively mild climatic phases of the Weichselian glaciation and that it was limited to summer periods because frozen ground prevented recharge in the winter.

D. B. SMITH
R. L. OTLET

UK Atomic Energy Authority,
Atomic Energy Research Establishment,
Harwell, Oxfordshire, UK

R. A. DOWNING
R. A. MONKHOUSE

Central Water Planning Unit,
Reading Bridge House, Reading, UK

F. J. PEARSON

United States Geological Survey,
Reston, Virginia 22092

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Processing structure of sentence perception

To extract meaning from a sentence, the listener must combine information from several levels of linguistic analysis. Psycholinguistic research¹ has documented a range of processing levels from the phonetic to the semantic, but has not explored the central problem of specifying the interactions between these levels. We have examined three fundamental questions about the processing structure of sentence perception. When, during sentence processing, does information at different levels become available to the listener; what is the time-course of the interactions between these levels; and what is the direction of information flow between them?

We used three speech monitoring tasks, in which the subject listens to a sentence and makes a timed response when he hears the target word. Identical monitoring, in which the subject knows in advance exactly which word to listen for, provides a baseline for assessing performance in the other two tasks. In rhyme monitoring, where the subject listens for a word that rhymes with the cue word given in advance, his response need only require a phonetic analysis of the target. But in category monitoring, where the target is a word belonging to a taxonomic category specified in advance, the response depends on a semantic analysis of the target. By placing these target words at different serial positions in the test sentences, we can measure the time course of different levels of processing.

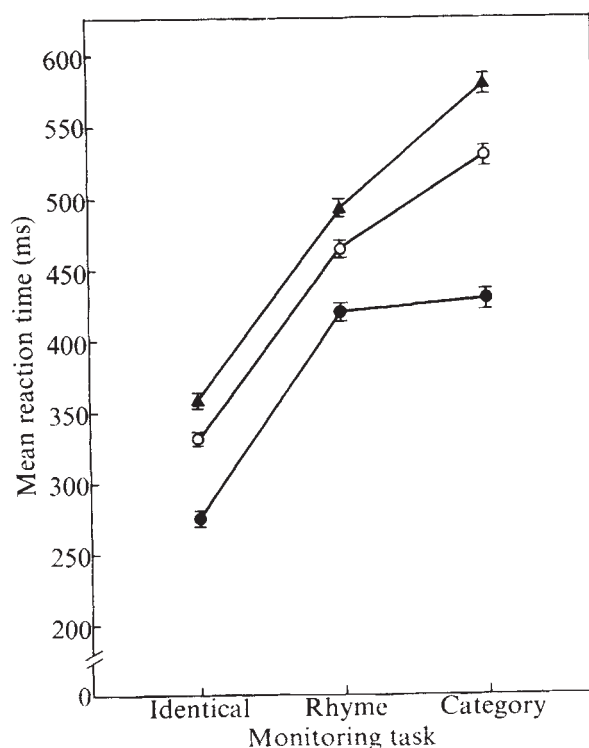
The three monitoring tasks were also co-varied with three

types of prose context—normal prose (NP), syntactic prose (SP), and random word order (RWO). If monitoring in an NP context is facilitated by the presence of sentence-level syntactic and semantic information, then response latencies will increase when there is no semantic interpretation available (in SP), and when syntactic information is also unavailable (in RWO).

Our earlier research^{2,3} leads us to propose an interactive parallel model of sentence processing, in which, from the first word of a normal sentence, the analysis of the input is conducted at all available processing levels. In particular, the information available at any one level of analysis can constrain and facilitate decisions at any other level, so that the continuing phonetic and lexical processing of each word is directly influenced by its current syntactic and semantic context³. As we will make clear in discussing the results, the predictions of this approach will contrast with those of serial theories, which assume varying degrees of delay before information at any one level of analysis can interact with information at any higher level^{1,4}.

The experiment, in summary, combines three monitoring tasks, three prose contexts, and nine word positions. Eighty-one target words were distributed equally across the second to the tenth word positions in the second sentence of 81 pairs of NP sentences. The same target words in the same word positions were also embedded in 81 SP and 81 RWO sentences. These materials were then assigned to nine versions, such that each of 45 subjects heard each of

Fig. 1 Overall monitoring response latencies (with standard errors indicated) for each monitoring by context condition. Each cell represents the mean of 405 observations, counterbalanced so that each subject, target word and word position contributed equally to each of the nine means. The SP sentences were constructed by replacing pseudo-randomly all content words in the NP sentences, except the target words, with new words of the same form-class and frequency. The RWO sentences were constructed by scrambling the word order of the SP sentences, keeping the serial position of the target word constant. The same target words occurred in each monitoring condition, but with different cue words given in advance. Thus, if the target word was 'cat', the cue word for identical monitoring was also 'cat', while in rhyme monitoring it was 'pat', and in category monitoring the category name 'animal'. ●, NP; ○, SP; ▲, RWO.



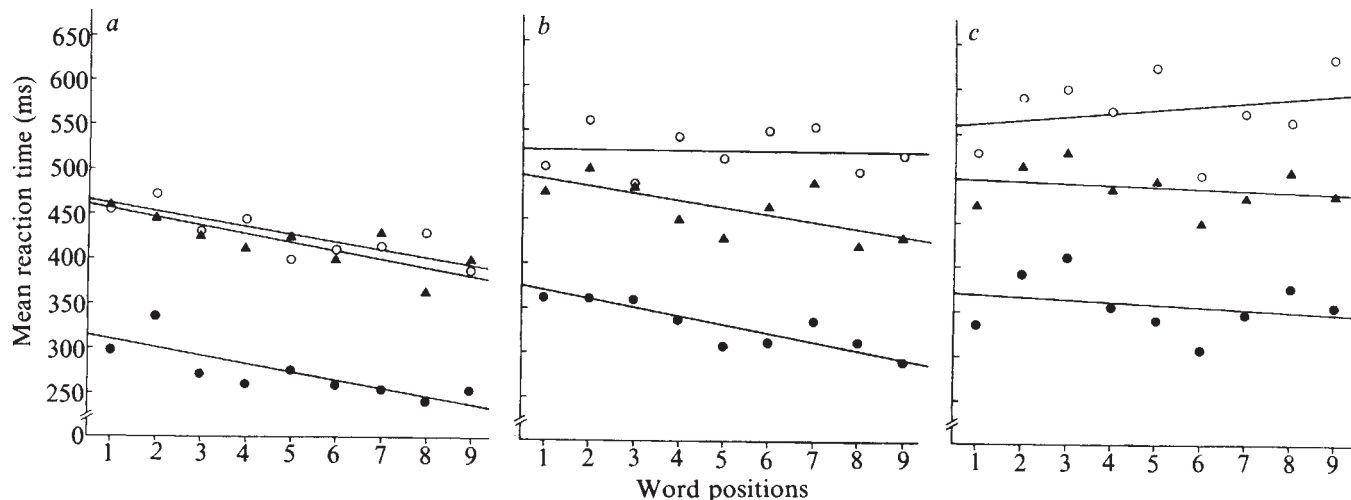


Fig. 2 Monitoring response latencies in each monitoring by context condition as a function of the word-position of the target word. Each point is the mean of 45 observations. Best-fitting least squares regression lines are drawn for each condition. Word-position 1 is the second word in the test sentence. *a*, NP; *b*, SP; *c*, RWO; ○, category; ▲, rhyme; ●, identical.

the 81 target words in one of the nine combinations of context by monitoring conditions.

The overall results are summarised in Fig. 1. Min F^1 values were computed⁵, using the untransformed raw data, with missing observations (less than 2%) replaced. The main effects of monitoring and of context, and the interaction between them, were significant ($P < 0.001$). Each of the means differed significantly from all other means ($P < 0.01$), with the exception of NP rhyme and category monitoring.

Monitoring latencies are faster in all NP conditions than in SP or RWO, and SP monitoring is similarly superior to RWO. This is predicted by the interactive parallel model, in which both semantic and syntactic information interact directly with continuing lower level decisions. On a serial model, lower level analyses are performed first, before being integrated with higher level information. Thus there should be no latency differences between contexts when the monitoring response could be based just on lower-level information—as in rhyme monitoring.

In addition, there are no differences between rhyme and category monitoring in an NP context, but large differences in SP and RWO. According to an interactive parallel model, semantically based decisions in NP can be just as fast as phonetically or lexically based decisions, since the listener develops a unified multi-level representation of the material as he hears it, and the higher and lower level attributes of this representation are equally accessible for making the monitoring response.

In contrast, any serial model has to predict longer latencies for category monitoring, since the defining characteristic of a serial model is that higher level decisions must, to some measurable extent, await lower level decisions. It should be noted that category monitoring in SP and RWO is much slower than rhyme monitoring, showing that the similarity between them in NP depends on the continuing semantic interpretation at the sentential level.

Effects of word position (Fig. 2) are found only in the conditions where hearing more of the sentence gives the subject additional information at the higher levels of analysis relevant to his responses. Latencies decrease as a linear function of word position in all NP monitoring tasks and in SP identical monitoring, with a weaker effect in SP rhyme. In particular, the goodness of fit and the slopes of the linear regression lines are remarkably similar for these major effects (Table 1).

Thus the advantage of NP over SP in identical monitoring is the same at all word positions. Similarly, within NP, the dissociation between identical and rhyme or category

Table 1 Regression of context by monitoring latencies on word position

Monitoring task		Prose context		
		NP	SP	RWO
Identical	$r =$	-0.78*	-0.87*	-0.21
	$b =$	-8.20	-8.62	-2.35
Rhyme	$r =$	-0.79*	-0.64†	-0.19
	$b =$	-8.18	-7.27	-1.68
Category	$r =$	-0.78*	-0.005	+0.25
	$b =$	-7.67	-0.05	+3.78

r , Pearson product-moment correlation coefficient; b , slope of linear regression line in ms.

* $P < 0.001$.

† $P < 0.05$.

monitoring is the same over word positions. This bears out the interactive parallel claim that analyses at all levels are initiated from the first word in a sentence. Although a serial model could predict differential decreases in latency for the later word positions, the differences between conditions should be minimal earlier in the sentence.

The data, therefore, consistently support an interactive parallel model. On the assumption that the monitoring task reflects the processes involved in ordinary listening, we conclude that a representation of the input at all available levels is initiated immediately in sentence processing, such that bi-directional and reciprocal interactions between these levels also begin very early in processing. The processing of spoken language, whether by humans or computers^{6,7}, may only be feasible when all available constraints, at whatever levels of analysis, are brought together during processing to limit the size of the search space within which the interpretation of the input is being established.

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WILLIAM MARSLER-WILSON
LORRAINE KOMISARJEVSKY TYLER

*Committee on Cognition and Communication,
Department of Behavioral Sciences,
University of Chicago,
Chicago, Illinois 60637*

Received June 6; accepted September 17, 1975.

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Determination of perspective reversals

MANY outline drawings of geometrical objects go through apparent reversals of perspective during prolonged viewing, and no adequate explanation for this is known¹. A classical explanation is that satiation or fatigue of the neural process which mediates the perception of one perspective leads to a replacement of the process by another one which has not been recently active²⁻⁴. I tested the satiation theory by studying whether the apparent reversals of perspective can be determined by adaptation to an unambiguous perspective seen in a stereogram or in a three-dimensional object. Adaptation blocked temporarily the appearance of the corresponding perspective in a reversible perspective figure.

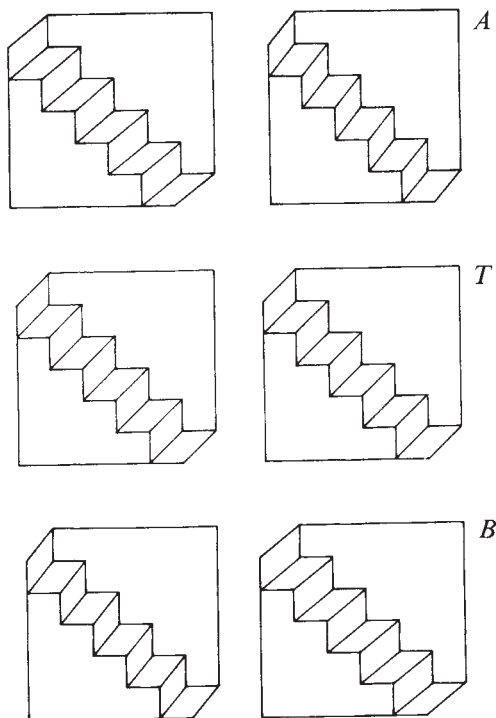


Fig. 1 Determination of perspective reversals through disparity specific adaptation. *A* and *B*, Adaptation stereograms with opposite disparities. *T*, Test stereogram without disparity. If reader stereoscopically fuses the test pair, an alternating perspective of the staircase is seen. Pairs *A* and *B* if properly fused produce a stereoimage which does not alternate in perspective. Adapt to *A* for a minute and observe then *T*: the apparent perspective of the test image now is opposite to that observed in *A*. Repeat the same by adaptation to *B* and notice that after adaptation to *A* or *B* the perspective of *T* is always opposite to the one observed in the adaptation figure.

The Schröder staircase served as the stimulus figure. The adaptation and test stereograms are shown in Fig. 1. There was no disparity in the test stereogram *T*. So its apparent perspective alternated: the staircase could be seen either from above (case *A*) or from below (case *B*). The adaptation stereograms (*A* and *B*) contained 3 mm of horizontal disparity so that pair *A* formed a stereoimage in which the staircase was seen from above and pair *B* formed an image in which the staircase was seen from below.

The stereograms were projected on to a screen with the aid of two timer-controlled projectors behind the screen. The subject viewed the screen at a distance of 57 cm, with his head held against a chin rest. A black divider extended

from the subject's forehead to the screen; hence, each eye saw only one half of the tangent screen. One half of the stereogram was shown to each eye. Prisms and lenses fitted into a spectacle frame corrected the retinal images of the figures so that a good fused image was formed. Subjects who were not able to form a stable stereoimage were rejected.

Black fixation crosses, one for each eye, were continuously present on the screen. With adequate fusion, the subject saw one luminous staircase figure and one black fixation cross in its centre. The fixation crosses were 6.4 cm apart and the size of the fused figure was 5.2 × 5.2 cm. The luminance of the stimulus lines was about 500 cd m⁻² and that of the background was 50 cd m⁻².

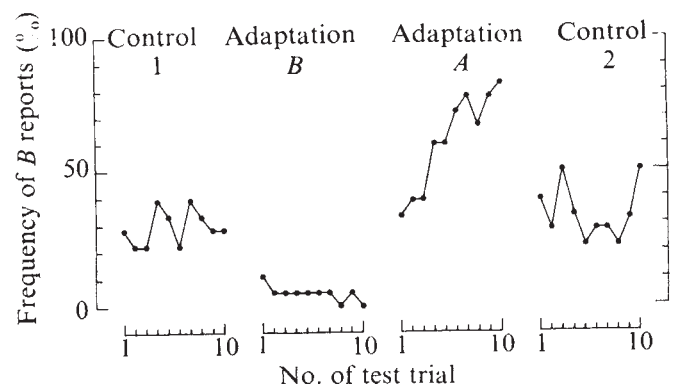
Four adaptation series were run, each consisting of ten trials. Each trial consisted of four events: (1) 5-s fixation to the adaptation figure, (2) a half second pause during which fixation was held, (3) one second fixation to the test figure, and (4) 10-s pause. The subject reported verbally or by means of a switch the apparent orientation of the staircase during its presentation. The test stimulus itself was used as the adaptation stimulus in the first and last adaptation series (controls 1 and 2). In the middle series, pairs *A* and *B* were used as adaptation stereograms, in alternating orders for different subjects.

Figure 2 shows the results for 18 subjects. When a stereogram without disparity served as the adaptation stimulus, 29.4% of all reports in control 1 and 33.3% in control 2 were case *B* reports. After adaptation to type *B* stereogram the average number of *B* reports fell to 4.6% and after adaptation to type *A* stereogram the number rose to 61.1%. Thus, it is clear that adaptation to a stimulus containing disparity led to a considerable decrease of reports of similar perspective in the test stimulus. The effect became stronger as a function of repeated adaptation. At the end of the ten-trial series 83% of subjects reported case *B* in the test figure after adaptation to *A* and no subject reported *B* after adaptation to *B*. Thus, 50 s of distributed adaptation time led to an almost complete determination of the perspective perceived in the neutral test figure.

The decay time of the adaptation effect was determined for 6 subjects by recording the apparent perspective of the test stimulus continuously. After a 1-min adaptation to a stereogram with disparity the average frequency of *B* reports returned to the unadapted level in about 20 s.

In some experiments, a three-dimensional Schröder staircase model was used as the adaptation stimulus. The

Fig. 2 Development of the disparity specific aftereffect during repeated testing after various forms of adaptation. Ordinate shows the percentage of subjects ($N = 18$) who saw perspective alternative *B* in the test figure which could be seen either as alternative *A* or alternative *B* during its 1-s presentation. Each test trial was preceded by a 5-s adaptation period. In control series the adaptation stimulus was the same as the test stimulus. In adaptation *B* the disparity of the adaptation stimulus evoked a perspective view of type *B*. Adaptation stimulus *A* evoked perspective *A*, respectively.



model was made of plastics and the edges of the model were black; the model produced projections like the ones shown in Fig. 1. Adaptation to the model prevented the visibility of the adapted perspective in a corresponding two-dimensional projection of the model. Thus, adaptation to a three-dimensional model produced the same result as adaptation to a stereogram.

These aftereffects are similar to other aftereffects in the third dimension⁵⁻⁷ in the sense that they are generated through adaptation to binocular disparity. They differ in the test stimulus in which the effects are observed. In the usual disparity-specific aftereffects the perceived depth of the test stimulus depends on actual disparity and the effect of adaptation can easily be understood through a change of perceived disparity. The present aftereffects are observed in the perspective of an object which is represented by a drawing; the apparent depth of the depicted object is not conveyed by means of disparity cues. The effect of adaptation in this case is paradoxical because adaptation to disparity affects a perceptual property which is not presented through disparity.

The results support the satiation theory of perspective reversals because they show that forced adaptation to one perspective view decreases the likelihood of continued perception of the same perspective. The role of binocular disparity in the adaptation effect is puzzling, however. It is evident that the satiation theory is not strictly testable as long as the neural processes mediating the impression of perspective in an object presented by a two-dimensional outline drawing remain unknown. The present results indicate that satiation taking place in disparity-specific neural mechanisms⁸⁻⁹ would be sufficient for inducing perspective reversals. The state of disparity-specific neural mechanisms seems to be one possible determinant of apparent perspective seen in drawings but the results do not imply that the perspective reversals ordinarily seen are caused by adaptation of these neurones.

VEIJO VIRSU

Psychological Laboratory,
University of Helsinki,
SF00170 Helsinki 17, Finland

Received June 26; accepted September 12, 1975.

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Is the aestivating lungfish the first vertebrate with suctional breathing?

THE current view is that ventilation of the lungs by aspiratory, negative-pressure inhalation probably occurred in the earliest, now extinct amphibians¹. But no aspiration breathing has been demonstrated in extant air-breathing vertebrates below reptiles, which use freely movable ribs and specialised muscles for this purpose. We report experiments showing that the aestivating lungfish relies on suctional breathing.

When the lungfish is in water the lung is ventilated by essentially aquatic breathing movements involving a buccal, positive-pressure swallowing of air, whereas expiration is largely effected by passive compression of the lung from the external water as the head is raised to the surface and the glottis opened². This basic mechanism of inspiration was passed on in only slightly modified form to the lunged amphibians. When the lungfish aestivates, however, in a soil-encased cocoon³, conditions for breathing are quite different. Air breathing alone can support its aerobic metabolism

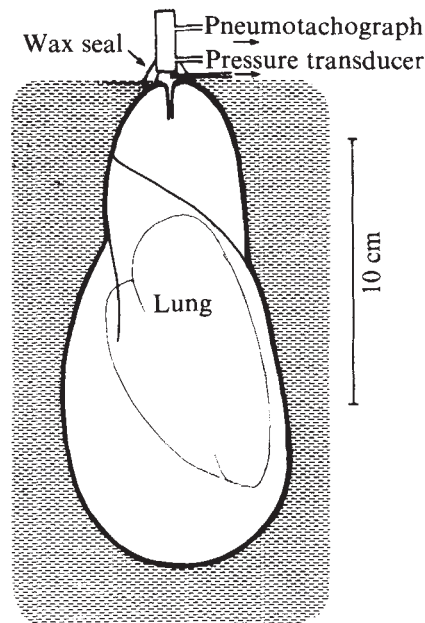


Fig. 1 Schematic representation of aestivating lungfish showing position of pneumotachograph tube and cannula for pressure measurements. The fish is covered by a cocoon (solid outline) and embedded in soil.

when, at the start of aestivation, the lungfish becomes enveloped by a hardened mucous secretion. This cocoon covers the entire surface and even enters the mouth to form a hollow, non-collapsible breathing tube connecting the bucco-pharyngeal cavity with the air channel in the soil leading to the outside air. This prevents the movements of the buccal floor, such as practised in the free swimming lungfish. More importantly, it prevents complete closure of the mouth and thus compels the animal to suspend positive-pressure buccal pumping for filling its lung.

The lungfish, *Protopterus amphibius*, Trewavas, used in our study were naturally aestivating and had been dug out of a dried up lake bed near Malindi, Kenya, 2 yr previously. Eight fish encased in the same soil in which they had entered aestivation, were transported by air to Aarhus where they were kept at 25 °C for 18 months before our study.

The breathing pattern of the fish was recorded continuously by pneumotachography (Statham-Godart) and pressure measurements (Statham) (Fig. 1). In addition, the soil surrounding some of the aestivating lungfish was removed carefully to expose the head and floor of the buccal cavity for visual observation of movements correlated with breathing. In one specimen a catheter was passed through the hollow breathing tube and into the buccal cavity to record pressure. Finally, one aestivating specimen was subjected to X-ray examination to reveal the size of the lungs and airways.

Figure 2 shows that normal breathing was periodic with a series of ventilatory movements alternating with apnoeic periods, which lasted from a few minutes to several hours. A ventilatory period always started with an inspiration. Inspiration was a two-phasic process—an initial phase of relatively rapid inflow (0.2 ml s⁻¹) ran, after a period of no air flow, into a second inspiratory phase when volume flow was less (0.1 ml s⁻¹) but lasted longer (Fig. 2). The longer lasting phase contributed most to the inspiratory volume, which typically totalled about 0.3 ml. The inspiratory phases lasted about 70% of the entire breathing cycle. The two phases of inspiration were attended by two cycles of negative (subambient) pressure recorded in front of the breathing tube. The second inspiratory phase ran directly into a single-phase expiration which was the most forceful of the breathing movements, resulting in air flows exceeding 1 ml s⁻¹.

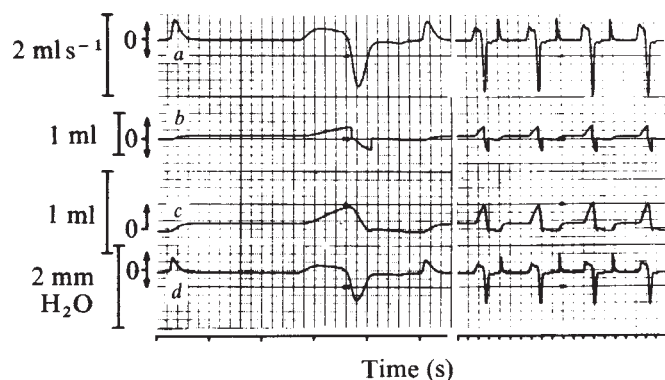


Fig. 2 Tracings showing: *a*, rate of inspired (upward deflection) and expired air flow, $\dot{v}$; *b*, inspired and expired volumes V_{I+E} ; *c*, inspired volume, V_I ; *d*, pressure at entrance of breathing tube, P .

A positive pressure (0.3 mm H₂O) more than twice the value of the negative pressure associated with inspiration correlated with the rapid expiration lasting about 10% of the breath cycle. The expired volume matched the combined volumes of inspiration in phases one and two. Thus both volumes inspired enter the lung and must do so as a result of suctional inhalation.

Pressure pulses recorded in the buccal cavity closely resembled those recorded in the air space in front of the breathing tube (Fig. 1). This is further evidence that air enters the lung by suctional attraction. If the first volume of air entering the lungfish had been positioned in the mouth for a second phase positive-pressure force filling of the lung, two positive-pressure phases, one attending the buccal force pump, the other the expiratory phase, should have been recorded from the mouth catheter. Further, if a positive buccal pressure filled the lung, no air flow should have been recorded through the pneumotach. As the recordings show, all pressure pulses were associated with air flow through the head of the pneumotach. Visual observation of the buccal floor during breathing showed no movements suggesting filling or emptying of the buccal cavity.

The X-ray study similarly showed a rather narrow air channel extending from the breathing tube through the buccopharynx to the lung. A buccal force filling the lung is thus also ruled out on this basis. The X rays revealed a surprisingly large lung shadow. A large lung volume in the aestivating lungfish was also suggested by the observation⁴ that a carefully excavated aestivating lungfish would float high when returned to water. Conversely, the breathing volumes recorded presently from aestivating lungfish are much smaller than those earlier estimated indirectly from non-aestivating fish⁵.

These data tend to support the hypothesis that suctional breathing probably evolved first in aestivating lungfishes. The mechanics of the breathing process and the activity of the associated muscles and skeletal parts are unknown.

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J. P. LOMHOLT
K. JOHANSEN

Department of Zoophysiology,
University of Aarhus,
DK-8000 Aarhus C, Denmark

G. M. O. MALOY

Department of Animal Physiology,
University of Nairobi,
Nairobi, Kenya

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Chemically-mediated host selection in a parasitic mite

MITES parasitise a broad spectrum of metazoans—molluscs, arthropods and vertebrates, including man. They transmit various pathogens to these hosts, and may cause severe medical and economic damage^{1,2}. Host preferences are commonly restricted to one or a few species³, and parasitism may result in profound ecological or biological effects, for example, the regulation of clutch size in birds⁴. Such effects have prompted some investigators to suggest the use of acarine parasites as natural control agents^{5,6}. In spite of all these factors, little is known about how mites select their hosts. We studied host discrimination in a typical acarine parasite, *Proctolaelaps nauphoetae* (Womersley)^{7,8}, and report that it uses a chemical sense to guide it to its only host, the cockroach *Nauphoeta cinerea* (Olivier).

Studies of other arthropod parasites (Hymenoptera) view host discrimination as three consecutive processes⁹⁻¹²: finding the host's environment, selecting an appropriate host, and accepting or parasitising this choice. Acarine parasitism seems to fit this pattern. In this report we focus on the mechanisms by which *P. nauphoetae* selects its host.

Mites use their first pair of legs as chemosensory organs^{1,2}. Ablation of these appendages renders *P. nauphoetae* incapable of finding a host¹³. Therefore we examined the attractiveness of potential chemical stimuli in two-choice preference tests. Material was applied to 7-mm paper disks and exposed to 15–25 mites in a specially designed chamber¹³. Each pair of stimuli in a test was matched in shape, size, texture, solvent and treatment so that the only difference between them was the material being tested. Mite distributions were determined after 3 h, and each experiment was replicated five or more times. The null hypothesis in all experiments was that mites would be equally distributed between the two choices. The G statistic was used to test the homogeneity of replicates and their goodness-of-fit to the null hypothesis. Probabilities cited below refer to the goodness-of-fit. Control experiments showed that mite movements were not influenced by chamber design ($P > 0.9$) or the presence of other mites ($P > 0.9$). Mites avoided white light ($P < 0.001$) but not red ($P > 0.9$); therefore all experiments were conducted under red illumination at constant temperature (27 °C) and humidity (50%). More than 12,000 mites were tested.

N. cinerea is a gregarious cockroach, forming colonies of several hundred individuals which live in close contact with one another and their own detritus¹⁴. As a result materials from faeces, expectorants, dead individuals and rotting debris are carried by each cockroach and could be used by mites as cues. We collected faeces from nine species representative of all phylogenetic lines of cockroaches¹⁵. Mites were attracted to all these ($P < 0.05$) except one, *Eurycotis floridana* (Walker), known to produce a strongly repellent compound¹⁶. Faeces from a laboratory rat were also attractive ($P < 0.01$), as was skatole ($P < 0.03$). These results suggest that mites are generally attracted to faeces, but do not rule out the possibility that additional attractive materials occur in host excrement. This was shown not to be the case: in a choice between host and non-host faeces, mites were equally attracted to both ($P > 0.95$).

In addition to faeces, mites showed a nonspecific attraction to legs excised from several cockroach species ($P < 0.01$), cockroach detritus ($P < 0.01$), and two compounds occurring in rotting organic material, putrescine and cadaverine ($P < 0.03$). These results suggest that *P.*

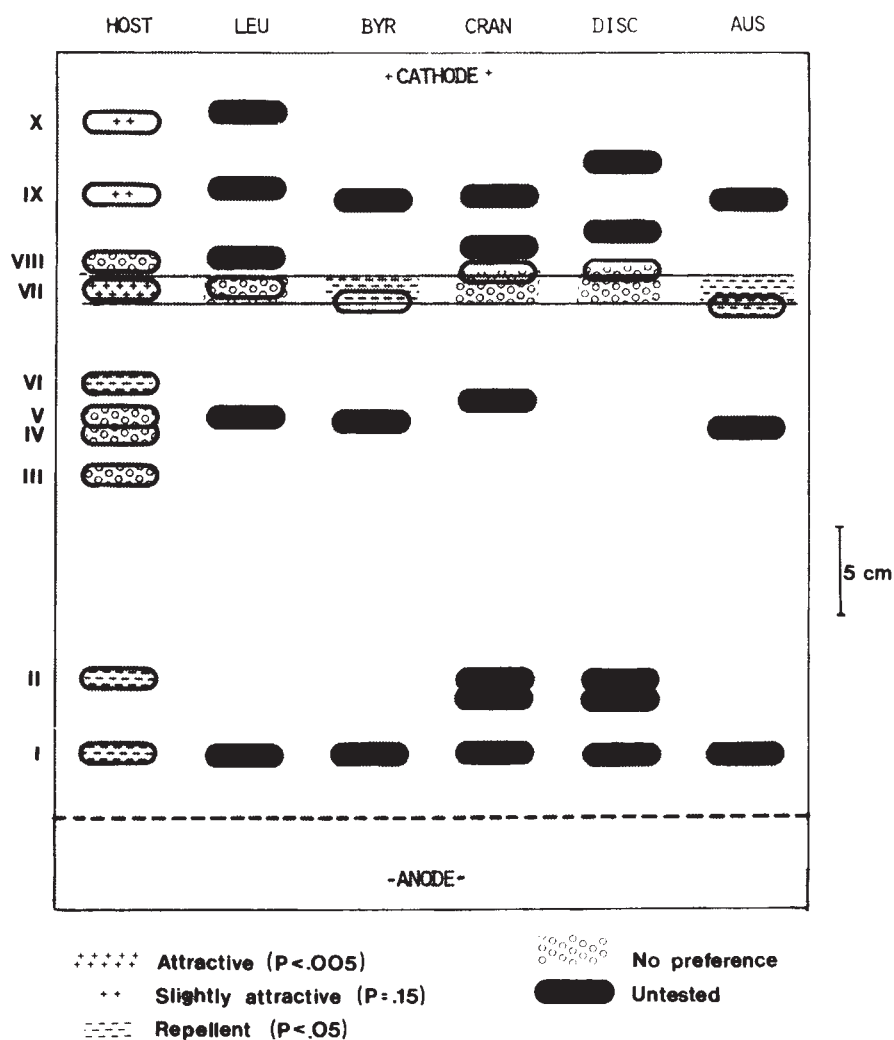


Fig. 1 Separation of nauphoetamine, a host-specific cue. Map of the components from cockroach extracts indicated by ninhydrin after high voltage paper electrophoresis (2–1/2 kV, 30 min, pH 6). Extracts were applied in equivalent concentrations on the dotted line after treatment with gel filtration. Mite responses to these components are indicated. Abbreviations: HOST, *Nauphoeta cinerea* (Olivier); LEU, *Leucophaea maderae* (Fab.); BYR, *Byrsotria fumigata* (Guérin); CRAN, *Blaberus craniifer* Burmeister; DISC, *B. discoidalis* Serville; AUS, *Periplaneta australasiae* (Fab.).

nauphoetae can recognise the proximity of potential hosts—a cockroach colony. To test this hypothesis, we isolated several hosts from their colony for 3 weeks, then tested them against colony-dwelling individuals. These mites categorically preferred the latter ($P < 0.001$); conversely, they were attracted to (but did not parasitise) a non-host cockroach (*Periplaneta australasiae* [Fab.]) that was permitted to live in a host colony for 3 weeks ($P < 0.01$).

P. nauphoetae showed a selective preference for cockroach expectorants rather than faeces and detritus. They were attracted by material produced by representative species in the host's family, Blaberidae ($P < 0.05$), but not by species in other families ($P > 0.9$). In addition, host expectorants were more attractive than host faeces ($P < 0.001$). These results suggest that expectorants, spread over a cockroach's body by grooming, could inform parasites that a potentially suitable host was near. Supporting this hypothesis is the result that 45 of 54 mites, observed while searching for a host, made a discrete and consistent change in their searching pattern as they neared a host.

Clean filter paper placed in a cockroach colony for several weeks became impregnated with material that strongly attracted *P. nauphoetae* ($P < 0.99$). In addition, their searching behaviour was essentially the same in the presence of either this paper or a live cockroach. Extraction of this paper with acetone, methylene chloride, ether, petroleum ether, methanol and ethyl alcohol failed to remove attractive material ($P > 0.9$). Aqueous buffers at a pH greater than 7 completely removed attractant ($P < 0.005$), and boiling destroyed its activity ($P > 0.99$).

The active extract was concentrated by lyophilisation and applied to a column of Sephadex G-50 calibrated for

polysaccharides¹⁷. Column effluents were pooled into three molecular weight fractions, concentrated, and tested for their attractiveness. Only one fraction (1,500–6,500 daltons) attracted these mites ($P < 0.005$). This active fraction was lyophilised, redissolved in pyridine buffer, pH 6, and electrophoresed on paper at 2.5 kV for 30 min. Half of this electrophoretogram was stained with ninhydrin for amines, *p*-anisidine for ketoses¹⁸, or Morgan–Elson reagents for hexosamines¹⁹. Ninhydrin indicated 10 cationic bands (Fig. 1). Of these, three bands—VIII, IX, and X—also reacted to the hexosamine test: there was no reaction to the ketose test. The other half of the electrophoretogram was cut into 0.25-cm² pieces and assayed in preference tests. Only band VII strongly attracted *P. nauphoetae* ($P < 0.005$); two other bands (IX and X) may have been weakly attractive ($P = 0.15$). We have observed the band patterns in Fig. 1 in 23 runs using five different extracts.

To determine whether the attractant isolated by electrophoresis is unique to the host, we collected material from five non-host species representing the major phylogenetic lines of cockroaches. These materials were extracted, gel filtered and electrophoresed exactly as was the host material. Electrophoretograms were marked with host extract, stained with ninhydrin, and material from non-host species occurring around band VII was offered to mites (Fig. 1). None of this material was attractive ($P > 0.9$), including one band with the same mobility as the marker; material from two species was slightly repellent. From these experiments we conclude that band VII from *N. cinerea* contains a host-specific attractant.

Further chemical analysis of band VII is planned. Preliminary results by ion exchange chromatography, thin-

Department of Zoology,
University of Texas,
Austin, Texas 78712

ROBERT H. BARTH

Department of Biological Sciences,
University of Maryland at Baltimore Co.,
Cantonsville, Maryland 21228

FRANK E. HANSON

Received July 8; accepted August 18, 1975.

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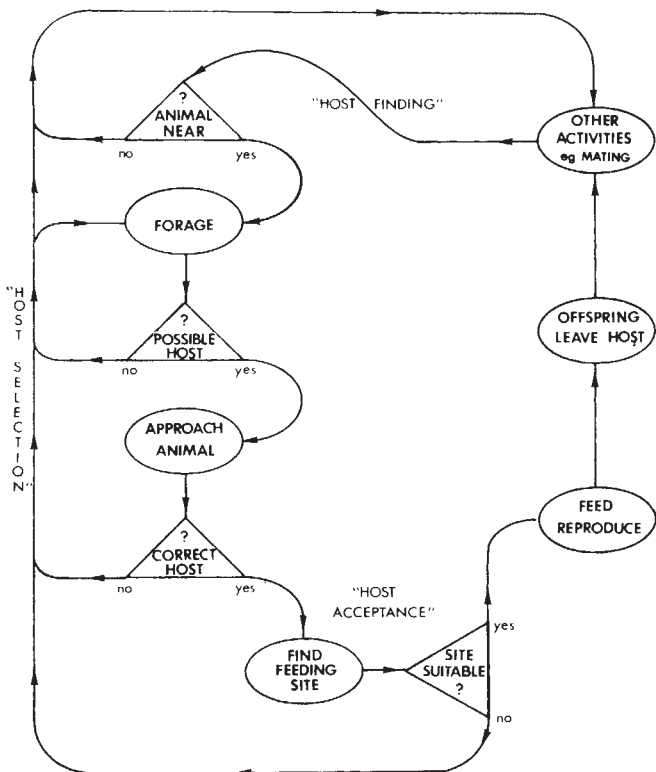


Fig. 2 A flow chart showing host discrimination. Host discrimination by *P. nauphoetae* is viewed as three consecutive processes indicated by "...". Ovals indicate simple activities while triangles represent behaviour requiring chemosensory discrimination.

layer chromatography, spectroscopy and spot testing suggest that it is a glycosaminoglycan, a poly-amino sugar. The only definite conclusion is, however, that band VII is a polar macromolecule which contains a primary or secondary amine and has a molecular weight between 1,500 and 6,500. Considering these facts, and the source of the material, we propose the name nauphoetamine.

Three chemically mediated levels of discrimination were used by these parasites. The probable relationship between mite behaviour and the process of host discrimination is shown diagrammatically in Fig. 2. The first level is host proximity for which faecal odour and colony detritus seem to be cues. Perception of these cues initiates a random, undirected search (foraging). In time, this activity brings it close enough to a cockroach (or other animal), to determine if it is a potential host—the second level of discrimination. Appropriate chemical cues, for example, cockroach expectorants, result in a direct orientation. In the final approach the third level of discrimination occurs: the parasite can determine through perception of nauphoetamine whether the cockroach is the correct species. If not, it resumes foraging or engages in some other activity. If so, it moves to the abdomen, grooms its legs, and attempts to find an unoccupied feeding site^{7,8}. If unsuccessful, the mite abandons the cockroach and continues to forage. If a feeding site is found, however, the process of host discrimination is consummated by attachment which may be followed by feeding and eventual reproduction.

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MICHAEL E. EGAN

Department of Biology,
Princeton University,
Princeton, New Jersey 08540

Cell within a cell or a circulating cell pair

I WISH to report that one of the coelomocytes of *Phascolosoma agassizii* Keferstein, 1866¹ (collected from Arroyo de Frijoles State Beach, California) which was previously considered to be one granulocyte is seen, under the electron microscope, to consist of two separate cells in a unique relationship.

The most unusual and distinctive feature of this cell pair is that there are two structurally separate cells, one within the other. In more than 100 electron micrographs of such cell pairs chosen at random, and in five pairs serially sectioned, no cell junctions, cytoplasmic bridges or points of fusion between the two cells were found.

The external cell (E) is a complete cell containing a nucleus (NE) and a scant rim of cytoplasm which surrounds the internal cell (Fig. 1). The cytoplasm of the external cell (Fig. 2) contains a Golgi complex (Gc), mitochondria, smooth and rough endoplasmic reticulum, free ribosomes and small electron-dense granules, as well as larger granules which are similar in appearance to vertebrate lipofuscin granules (Lf). At high magnification, small fenestrations 0.018 μ m wide are occasionally distinguishable along the external plasma membrane (arrows). This may be an artefact resulting from the plane of sectioning of the irregular buffy-coated outer membrane.

The external cell is separated from the complete internal cell by an extracellular space averaging 0.07 μ m wide filled with a fibrillar reticulum (Fig. 2).

The cytoplasm of the enclosed cell is almost filled with large granules of various densities. The outer granule membranes are often lined with ribosomes (not visible in the fields shown). The nucleus of this cell (NI) is displaced to a site near the border of the external cell. The organelles—a Golgi complex, mitochondria (M), rough endoplasmic reticulum, free ribosomes, microtubules and glycogen (Figs 1 and 2)—are concentrated around the nucleus where new granules can often be seen forming from the Golgi complex.

I observed no granules being extruded from the cells or disruption of the granules during clotting of coelomic fluid. But during routine processing for electron microscopy, the

granules of the enclosed cell often disrupted, leaving the associated cell, reticulum fibres and enclosed cytoplasmic membrane. No cells were observed in mitosis and the GAC (granulocyte with its associated cell) was non-phagocytic.

Of the four morphologically distinguishable cell types found in the coelomic fluid of *P. agassizii* (omitting gametes) the cell within a cell was the least common. For every three ciliated urns, 30 granulocytes and 566 haemocytes, there was only one GAC (counts taken from 25 pooled blood samples, four worms each). The GAC ranged in size from $26\mu\text{m} \times 15\mu\text{m}$ to twice this length and width. In preparations of whole cells stained with Wright's blood stain two nuclei were seen per GAC. The nuclei were observed in all possible positions relative to each other, from close together to opposite poles (Figs 1 (inset) and 2).

In paraffin sections, 4–6 μm , the granules in the enclosed cell exhibited two staining reactions with periodic acid-Schiff (PAS), Giemsa and trichrome stains². They were either PAS positive or negative; PAS-negative granules stained orange with PAS orange G. With Giemsa the granules stained acidophilic or basophilic, and with trichrome, they stained red or green. One cell sometimes exhibited granules with both staining properties although homogeneously stained granules were the most common form. No histochemical stain differentiated the internal granulocyte from its external associated cell.

When 0.5- μm sections embedded in Araldite and stained with Toluidine blue were viewed by light microscopy, the external associated cell was not visible unless some internal granules had been disrupted. In most GACs, the granules of the internal cell obscured all other cytoplasmic features (inset, Fig. 1).

In unfixed preparations of whole cells, the GAC was reactive for both lipase³ and alkaline phosphatase⁴. Light microscopy indicated that all large internal granules were positive for lipase whereas the alkaline phosphatase reaction was random, often appearing more prominent around the periphery.

A circulating cell pair is an unusual finding. There is no report in the literature on invertebrates. Emperipolesis—cells within cells—has been reported in tumour cells⁵ of vertebrates and as a transitory state for migrating lymphocytes passing through the endothelial cells of post-capillary venules in lymph nodes⁶.

A diverse population of granulocytes has been observed

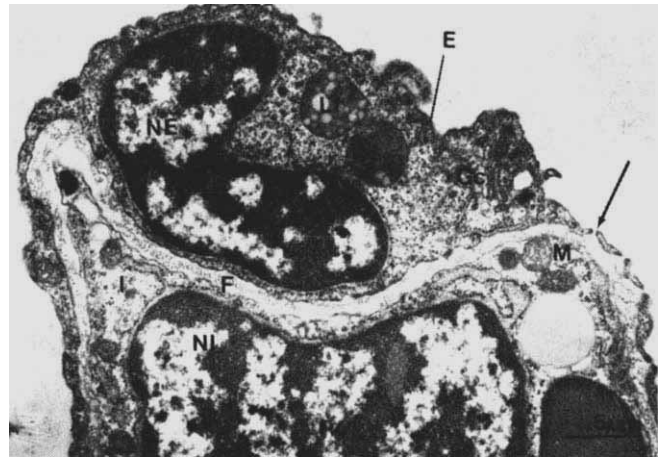


Fig. 2 Electron micrograph of the nuclear area of the GAC. Reticular fibrils can be seen separating the two cells. Lf, lipofusein granules; M, mitochondrion; F, fibrillar reticulum. Other labels as for Fig. 1.

in various sipunculid species^{7–11}. One group of granulocytes is described as multinucleate, larger with larger granules and differing in its acidophilic staining and non-phagocytic nature; but using electron microscopy it has been shown that in *P. agassizii* these are actually two associated cells. The different histochemical properties of this cell type and occasionally within a single cell with either the PAS, trichrome or Giemsa stain, could indicate different stages in packaging and condensation of a mucoprotein granule product. Variations in density revealed by electron microscopy, and the formation of new granules from the Golgi complex show a developmental sequence in the granule population. The purpose of the granules of the internal cell and the special conditions under which they might be released is not known. The granulocyte with associated cell could be a regulatory cell. The associated cell with the extracellular reticulum appears to be a physical support for the large internal granulocyte. Perhaps changes in the coelomic fluid cause changes in the external cell, resulting in lysis of the support structure and release of the internal cell's granules.

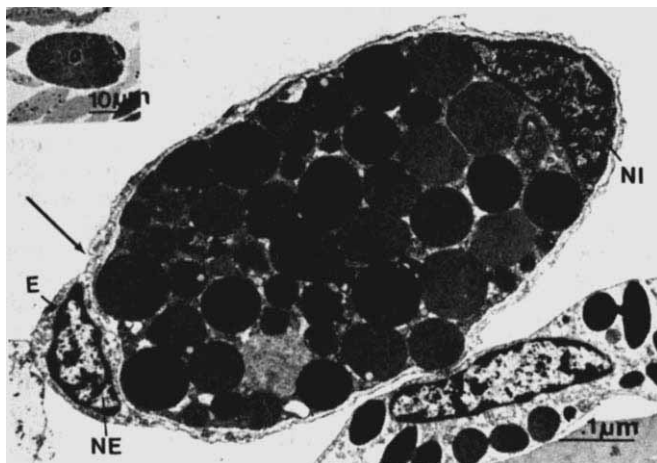
LINDA DYBAS

Universität Ulm,
Abteilung für Pathologie I,
79 Ulm (Donau), Oberer Eselsberg, FDR

Received July 16; accepted August 29, 1975.

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Fig. 1 Electron micrograph of a typical GAC. The cytoplasm of the inner cell, the granulocyte, is packed with dense granules. The associated cell is composed of a nucleus with scant cytoplasm completely surrounding the granulocyte. E, external cell; NE, nucleus of external cell; I, internal cell; Gc, Golgi complex; NI, nucleus of internal cell. —> Fenestration of external plasma membrane. Inset, toluidine-blue-stained Araldite section of a typical GAC.



Superior growth of the right gonad in human foetuses

RIGHT and left mammalian gonads do not usually differ noticeably either in size or development, in contrast to the situation in birds, where ovarian development is usually confined to the left side¹. Nevertheless, when the differentiation of the gonads follows an abnormal course, lateral asymmetry becomes apparent also in mammals. In human hermaphrodites, who have a testis as well as an ovary, the testis is preferentially situated on the right and the ovary on the left side^{2–4}. This

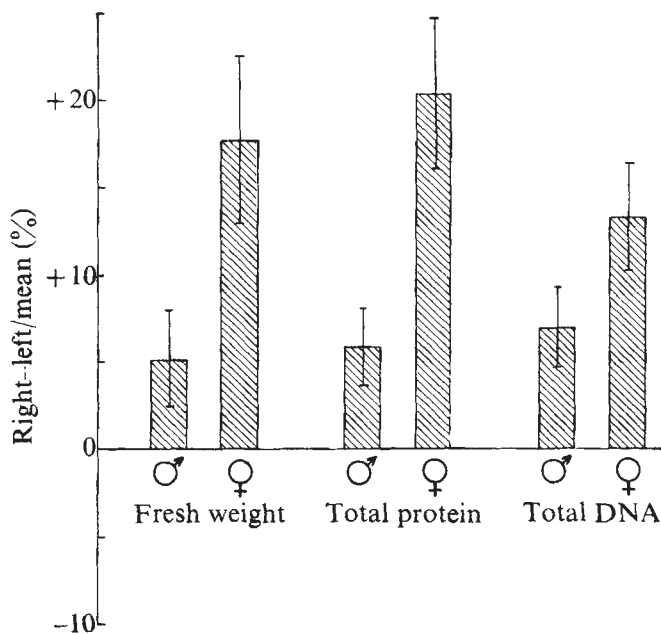


Fig. 1 Difference in fresh weight, total protein and total DNA contents between the right and left gonads of 14 male and 9 female foetuses aged between 20 and 28 weeks (estimated from crown-rump lengths). Bars represent the sums of the differences between right and left gonads, divided by the mean gonadal value, expressed in %; vertical lines represent $\pm$ s.e. After removal, the gonads were stored in 0.03 M phosphate buffer, pH 7.0 at -15°C . Before analysis, pairs of gonads were thawed out and trimmed of extragonadal material under a dissecting microscope. Trimmed gonads were blotted dry on filter-paper, weighed and ground in the above phosphate buffer using a loose glass-glass homogeniser. Samples of the ground material were assayed in duplicate for protein (using the procedure of Lowry *et al.*¹²) and for DNA (as described by Burton¹³). Bovine albumin (Sigma) and herring sperm DNA (Sigma) Type IV were used to calibrate the protein and DNA assays respectively.

finding is independent of the chromosome constitution^{3,4}. Also, certain gonadal tumours are known to be more frequent on the right side; this applies to dysgerminomas, which are seminoma-like tumours of the ovary⁵. The question thus arises whether mammalian gonads exhibit an inherent but undetected asymmetry between right and left sides. To answer this question, we have compared right and left gonads from therapeutically aborted human foetuses, using the criteria of fresh weight, total protein and total DNA contents.

Figure 1 shows that when the values for left gonads are subtracted from the corresponding values for right gonads, the difference is positive for all three criteria in both males and females. We can thus conclude that the right gonad is superior to the left in both protein and DNA contents, and thus, one can confidently assume, in cell number. This difference seems to be greater in females than males. The larger size of the right compared with the left ovary has previously been reported in adult British horseshoe bats of the genus *Rhinopholus*, in which the follicles of the left ovary fail to mature⁶.

An increased growth rate of the right mammalian gonad, at least during part of its development, could explain the greater risk of some gonadal tumours developing on the right compared with the left side. Moreover, the preferential situation of testes on the right and ovaries on the left side in patients with hermaphroditism²⁻⁴ may be accounted for simply by the asymmetrical growth of the two gonads in normal mammalian foetuses.

Foetal testes grow markedly faster than ovaries in rats⁷ and mice⁸. We have observed a similar effect in human foetuses younger than 25 weeks. The mean gonadal weight of 10 male foetuses (average age 21.5 weeks) was 41.9 mg and that of 6 female foetuses (average age 22.0 weeks) was 25.3 mg, the difference being significant at the 1% level.

Usually the differentiation of gonads into testes and ovaries

is firmly controlled by the sex chromosome constitution, but this mechanism clearly fails to operate during the development of hermaphrodites. In this situation the differentiation of the gonads will be determined by local conditions, of which body side is one of the determining factors. Gonads situated on the right tend to become testes while those on the left are more likely to develop into ovaries. Our demonstration that the right gonad is normally the larger one seems to offer strong evidence in favour of a causal relationship between increased growth and testicular differentiation in mammals^{9,10}.

It is interesting that in chick embryos of both sexes, the left gonad is larger than the right¹¹. Since in birds the female is heterogametic with XY(ZW) sex chromosomes, in contrast to the situation in mammals, it seems that birds are a mirror image of mammals both as regards gonadal asymmetry and their sex chromosome constitution.

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URSULA MITTWOCH
DAVID KIRK

Department of Human Genetics,
University College London,
Wolfson House,
Stephenson Way, London NW1 4HE, UK

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Low levels of genetic heterozygosity in Hymenoptera

It has been suggested¹⁻⁴ that organisms with a haplodiploid sex determination system should have reduced genetic variability relative to diploid organisms. In haplodiploid systems such as in bees and wasps (Hymenoptera), unfertilised eggs develop into haploid males and fertilised eggs into diploid females. Thus the frequency with which alleles are exposed to selection in an effectively homozygous state is increased over comparable diploid species. A lower level of genetic variation would be expected for haplodiploid organisms, relative to diploid organisms, for that genetic variation which is not neutral and is expressed in the haploid sex. Thus, measurement of the level of genetic variability in haplodiploid species is of considerable interest in understanding the mechanisms responsible for maintaining the high levels of genetic polymorphism in diploid organisms^{5,6}. For this reason, we have measured the genetic variability in haplodiploid species using the combined techniques of electrophoresis and histochemical staining^{7,8}. As predicted by selection theory, we have shown that the Hymenoptera (haplodiploid organisms) have a lower level of genetic heterozygosity and a lower frequency of enzyme polymorphism than do diploid organisms.

Table 1 Allelic frequencies for enzyme loci resolved for seven species of solitary Hymenoptera

Enzyme* locus	<i>Stictia carolina</i> (Fabricius)	<i>Chalybion</i> <i>californicum</i> (Saussure)	Wasps <i>Sceliphron</i> <i>caementarium</i> (Dury)	<i>Scolia dubia</i> <i>dubia</i> (Say)	<i>Trypargilum</i> <i>politum</i> (Say)	Bees <i>Nomia</i> <i>heteropoda</i> (Say)	<i>Savastrea</i> <i>obliqua</i> (Say)
3 HBDH	0.91 0.06 0.01 0.02 1.00	0.28 0.64 0.08	—	—	1.00	1.00	1.00
HPI	0.02	1.00	—	1.00	—	1.00	1.00
MDH-1	0.98	—	1.00	0.02	1.00	1.00	1.00
MDH-2	1.00	0.07 0.93	0.77 0.23	0.98 1.00	1.00	1.00	1.00
MDH-3	—	—	—	—	—	1.00	0.90 0.10
GAPDH-1	1.00	1.00	—	1.00	1.00	1.00	1.00
GAPDH-2	1.00	—	—	1.00	1.00	—	—
G3PDH-1	1.00	0.01 0.99	1.00	1.00	1.00	1.00	1.00
G3PDH-2	1.00	1.00	1.00	1.00	0.47 0.53	1.00	1.00
G3PDH-3	—	—	—	1.00	1.00	—	1.00
LDH	0.91 0.09	1.00	1.00	—	0.93 0.07	1.00	0.03 0.97
GDH	1.00	1.00	1.00	—	1.00	1.00	1.00
G6PDH	—	—	—	—	0.04 0.96	0.13 0.17 0.70	0.07 0.06 0.03 0.84 0.97
PGM-1	0.02 0.98	—	1.00	1.00	0.91 0.09	—	0.03 1.00
PGM-2	1.00	—	1.00	1.00	1.00	—	—
6 PGDH	0.40 0.60	0.98 0.02	—	0.10 0.90	0.07 0.91 0.02	—	—
IDH-1	1.00	1.00	1.00	1.00	1.00	1.00	1.00
IDH-2	1.00	1.00	1.00	—	1.00	—	—
FO	1.00	1.00	—	—	—	—	—
EST-1	0.03 0.97	1.00	1.00	0.65 0.35	1.00	0.55 0.32 0.13	1.00
EST-2	—	0.86 0.08 0.06 0.14	0.52 0.36 0.12	0.04 0.96	0.04 0.96	1.00	—
EST-3	—	0.86 1.00	—	1.00	1.00	1.00	—
EST-4	—	—	—	—	—	—	—
Alleles sampled	120	103	107	92	68	60	60
Number of loci	17	16	12	15	19	15	16
Frequency of polymorphic loci	0.35	0.37	0.17	0.27	0.32	0.13	0.25
Mean heterozygosity	0.056	0.073	0.078	0.051	0.059	0.070	0.038
S.e.m. heterozygosity	0.029	0.036	0.055	0.032	0.028	0.048	0.021

Allelic frequencies for enzyme loci resolved for seven species of solitary Hymenoptera. Although many precedents indicate that specific enzyme phenotypes represent at least one locus, in the absence of genetic crosses or allelic variation, it is difficult to determine whether each isozyme observed is coded in a separate locus. We have therefore been conservative in our estimates. The numerical designation assigned to multiple alleles and multiple loci coding the same enzyme function are based on their relative electrophoretic mobility with '1' being the most anodal. Enzyme (and coenzyme) used: 3 HBDH, 3-hydroxybutyrate dehydrogenase (NAD); HPI, hexosephosphate isomerase; MDH, malate dehydrogenase (NAD); GAPDH, glyceraldehyde-phosphate dehydrogenase (NADP); G3PDH, glycerol-3-phosphate dehydrogenase (NAD); LDH, lactate dehydrogenase (NAD); GDH, glutamate dehydrogenase (NAD); G6PDH, glucose-6-phosphate dehydrogenase (NADP); PGM, phosphoglucumutase; 6 PGDH, 6-phosphogluconate dehydrogenase (NADP); IDH, isocitrate dehydrogenase (NADP); FO, formazan oxidase (tetrazolium oxidase); EST, esterase (nonspecific esterases).

The results of this study for seven species of Hymenoptera are shown in Table 1. Each population represented was collected during a single day at one locality. The mean frequency of polymorphic loci and the mean heterozygosity per locus for the haplodiploid species is significantly lower than those for diploid organisms (Table 2). These results are consistent with substantial portions of observed enzyme variation being selectively not neutral. In addition to detecting selection against specific allelic isozymes, we may also be detecting the results of selection against other genes which are closely linked to those coding the allelic isozymes investigated. Other factors, such as small population size and inbreeding, could depress levels of polymorphism. This explanation is made less likely, however, by the fact that

the Hymenoptera species investigated are solitary and powerful flyers found over wide geographical areas. Partial replicates of the data in Table 1 were done on separate populations of *Sceliphron caementarium*, *Scolia dubia* and *Trypargilum politum*. No significant inbreeding or inter-population differences were found.

It is interesting that the variation in mean heterozygosity for the seven Hymenoptera species representing two superfamilies and seven genera is less than that for seven species within the single genus *Drosophila* and, in addition, is less than that for four species from the rodent genera *Mus* and *Peromyscus* (Table 2). It has been argued that the more homogeneous an environment, the smaller the store of genetic variation maintained⁹⁻¹¹. For our data to be

Table 2 Values for the average frequency of polymorphic loci and average mean heterozygosity for seven Hymenoptera species and thirteen diploid organisms

	Average frequency of polymorphic loci	Average mean heterozygosity
Hymenoptera (from Table 1)	$\bar{X} = 0.266$ s.d. = 0.090 s.e. = 0.034	0.061 0.014 0.005
Diploid organisms*	$\bar{X} = 0.392$ s.d. = 0.185 s.e. = 0.051	0.101 0.039 0.011
<i>Drosophila</i> species*	$\bar{X} = 0.507$ s.d. = 0.184 s.e. = 0.070	0.125 0.035 0.013
<i>Mus</i> and <i>Peromyscus</i> species*	$\bar{X} = 0.255$ s.d. = 0.048 s.e. = 0.024	0.079 0.027 0.013

*Data taken from R. C. Lewontin⁹.

These values were calculated separately for both seven species of *Drosophila* and four species of *Mus* and *Peromyscus*.

consistent with this theory, it would be necessary for the variation in the environmental heterogeneity to be less for the order Hymenoptera than within the genera *Mus* and *Peromyscus* or *Drosophila*.

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ROBERT A. METCALF*

Department of Biology,
Harvard University,
Cambridge, Massachusetts 02138

JOHN C. MARLIN

Department of Entomology,

GREGORY S. WHITT†

Department of Genetics and Development,
515 Morrill Hall,
University of Illinois,
Urbana, Illinois 61801

Received July 28; accepted September 5, 1975.

* Present address: Department of Zoology, University of California, Davis, California 95616.

† To whom reprint requests should be addressed.

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Inheritance of juvenile shell colour of the oyster drill *Urosalpinx cinerea*

MARINE prosobranchs have been extensively studied to elucidate the origin of their shell colour. Some authors have indicated that shell colour is in the main environmentally determined, that is, by diet^{1–5}, by light⁶, or by salinity⁷. Colour also results from direct genotypic action, for example, the change to adult coloration with maturity in some *Cypraea* species^{8,9}, or the restoration of initial colour patterns after shell repair^{10,11}.

Studies of colour-morph frequencies in natural populations of marine prosobranchs usually make the implicit assumption that colour characteristics are inherited^{12–21}, but it can be argued that the characteristic(s) used are not under close genetic control. Without information from breeding studies, the occurrence of environmental factors, which may affect the results, cannot be assessed. A more appropriate strategy would be to know the

inheritance of a characteristic before attempting large scale field studies.

In the oyster drill *Urosalpinx cinerea* adult shell colour grades from brown to purple-grey, with dark purple and white shells also occurring. Juvenile shells (<15 mm shell length (SL), measured from the siphonal canal tip to the spire apex), however, appear somewhat more discontinuous in colour. Young hatching from capsules deposited in the laboratory are either brown, purple, or albino²² (M. R. Carriker, personal communication). This report describes results of laboratory breeding experiments which indicate that juvenile shell colour is inherited in a manner which can be explained using a triallelic, single-locus genetic model, with a dominance hierarchy among the alleles.

In September, 1972, young *U. cinerea* (<8 mm SL) were isolated from local populations. These animals were raised in individual isolation until the shell length was about 15 mm when they were sexed²³, marked²⁴, and combined in pairs. Pairs were reared in separate cages, and were completely immersed in heated, flowing, seawater (system of Carriker and Van Zandt²⁴) at a temperature of at least 15 °C. *Mytilus edulis*, collected from sites where *Urosalpinx* does not occur, were provided as food; thus no extraneous drills were introduced into the cages.

Egg capsules were allowed to develop in the parents' cage until tinges of pigmentation appeared in the developing shells. The capsules were removed and placed in a hatching cage (screened with stretched nylon hose; average mesh 0.5 mm). Daily sprayings prevented clogging of the screens. As young emerged from their capsules, they were removed and placed in a second, identical cage with mussels (<3 mm valve length). This precluded cannibalism of newly emergent and emerging drills by already emerged siblings.

As the young grew, increasingly larger mussels were required, producing waste-removal problems in the fine-meshed cages due to low water flux. Some of the first groups of young consequently suffered extensive mortality from water fouling. Transferring the young to larger-meshed cages (1.5 mm) as soon as they were large enough to be retained (about 4 weeks after hatching) markedly decreased this mortality. Several groups of young were reared from crosses which suffered extensive mortality; usually only the first group was reared in more successful crosses.

Colour scoring of the external shell colour of newly emerged snails was carried out in seawater under a dissecting microscope, using incandescent lighting. Fourteen pairs produced an F₁ generation. Four crosses (A14, A15, A16 and A18) provided capsules in September, 1973; the remainder began ovipositing in February, 1974.

Three discrete external juvenile shell colours were observed among hatching *U. cinerea*: purple, brown and white. The data obtained were consistent with a triallelic, single-locus genetic model with complete dominance in the order purple > brown > white (Table 1).

With growth, however, subtle shell colour changes occur. For instance, purple bands normally occurring on interior surfaces of *Urosalpinx* shells, sometimes penetrate the shell and appear on the external surface, producing a brown, purple-banded snail, if the bands remain narrow. If the bands diffuse, a brown juvenile becomes a purplish adult. This external expression of normally interior bands is inherited, but more work is needed to establish the exact mode.

Additionally, a purplish hue is generally incorporated with age into the shells, eventually obscuring the juvenile colour. This may result either from genetic modifiers, or from prolonged mussel feeding, as suggested originally by Moore². The contribution of diet to adult shell colour is being investigated.

Prosobranchia in general can be split into two groups by the acid-extractability of their shell pigments. Archaeogastropod pigments which are protein-bound (chromoproteins) resist this procedure²⁶. Pigments elaborated by archaeogastropods are probably either digestive residues or end-products of aborted synthetic pathways^{26,27}. That archaeogastropod shell colour changes with diet^{1,3,5} is thus not surprising.

Table 1 F₁ colour morphs produced by the 14 *Urosalpinx cinerea* pairs

Cross	Parental/ juvenile shell colour	Colour of F ₁			Total hatched	χ^2	Probability
		Purple	Brown	White			
A3	P × B	60 (63.5)	67 (63.5)	—	127	0.38	0.9 > P > 0.5
A4	B × B	—	108 (108)	—	108	—	—
A6	P × B	23 (23)	23 (23)	—	46	0.00	0.9 > P > 0.5
A8	B × B	—	81 (81)	—	81	—	—
A9	P × P	96 (97.5)	34 (32.5)	—	130	0.09	0.9 > P > 0.5
A10	P × P	73 (70.5)	21 (23.5)	—	94	0.35	0.9 > P > 0.5
A11	B × B	—	93 (93)	—	93	—	—
A12	B × B	—	119 (119)	—	119	—	—
A13	B × P	123 (116.5)	110 (116.5)	—	223	0.72	0.5 > P > 0.1
A14	B × P	40 (39)	38 (39)	—	78	0.05	0.9 > P > 0.5
		50 (46.5)	43 (46.5)	—	93	0.52	0.5 > P > 0.1
A15	B × W	—	28 (23.5)	19 (23.5)	47	1.72	0.5 > P > 0.1
A16	P × P	70 (72.9)	27 (24.3)	—	104	0.41	0.9 > P > 0.5
A17	B × W	—	45 (49.5)	53 (49.5)	99	0.65	0.5 > P > 0.1
A18	B × B	—	95 (95)	—	95	—	—

Female genotypes are listed first in the parental/juvenile shell colour column. Only the major juvenile colour allele is given. Because of observed dominance relationships, and without F₂ colour segregation information, recessive alleles could not be definitively established in all crosses. Thus no recessive allelic assignments were made. Expected frequencies (in parentheses) were calculated assuming a single-locus, triallelic segregation model. Two groups of young were reared in cross A14 as the first batch suffered considerable mortality.

Meso- and neogastropod shell colour, however, is not totally independent^{2,4} of diet. *Polinices duplicatus* lightens the colour of its callus with a dietary shift towards increased consumption of *Mya*, but the rest of the shell remains unchanged⁴. Moore concluded that shell-banding in *Thais* results from a mussel diet². Berry and Crothers¹⁷ suggested that the capacity for band expression in *Thais* is genetically controlled, analogous to that of terrestrial pulmonates²⁸⁻³¹. Whether or not a *Thais* becomes banded after feeding on mussels thus depends on its inherited susceptibility. This illustrates the intimate relationship between genotypic and environmental components which influence prosobranch shell colour.

In *Urosalpinx*, the inherited juvenile ground colour can be modified with growth by both genetic (shell-band expression; hue modifiers) and environmental (diet) factors. The degree that each component predominates seemingly depends on the prevalence of certain dietary items. Clearly, care must be exercised in interpreting results of studies of shell colour of marine prosobranchs which assume close genetic control. Controlled breeding experiments are essential as a first step towards unravelling the complex and interrelated mechanisms that produce such morphological characters.

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TIMOTHY J. COLE

Boston University Marine Program,
Marine Biological Laboratory,
Woods Hole, Massachusetts 02543

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Acrosomal chromocentre in newt spermiogenesis

STUDY of the distribution of satellite DNA during the later stages of spermiogenesis led to a model in which chromosomes of the mature sperm nucleus are U-shaped with posterior centromeres^{1,2}. We have now studied a closely related chromosome marker—constitutive heterochromatin—and, using C banding techniques³, have found a polarised arrangement of chromocentres in spermatids of several newt species.

Adult *Triturus alpestris*, *T. cristatus* and *T. vulgaris* were captured from their natural habitat near Ulm, south-western Germany; *T. marmoratus* was caught in southern France (Banyuls-sur-Mer) and *Diemyctilus viridescens* was obtained from a dealer. Four hours before they were killed, the animals received an intraperitoneal injection of 0.5 ml of a 3% (w/v) colchicine solution. Testes were cut into small pieces and exposed to hypotonic conditions in distilled water for 30 min. Shortly before centrifugation of the suspension, 0.1 of the volume of 1 mM CaCl₂ solution was added, to prevent clumping. Slides were fixed and prepared according to Evans *et al.*⁴. Only 15 s of denaturation in 0.07 N NaOH was needed to produce C bands by the method of Arrighi and Hsu⁵.

As in many species, including man⁶, chromosomes of *Triturus* have segments of constitutive heterochromatin chiefly in their pericentric regions⁶. Figure 1 shows heterochromatin segments on both sides of the centromeres of chromosomes in spermatogonial metaphase and of bivalents at diakinesis of spermiogenesis in *T. vulgaris*. These segments should also be stainable by C banding at later stages of spermatogenesis.

The typical arrangement of chromocentres in spermatids is shown in Fig. 2. There was conspicuous asymmetry: most of the large, irregularly shaped heterochromatin particles were in the half of the nucleus which later becomes the posterior pole. One large chromocentre,

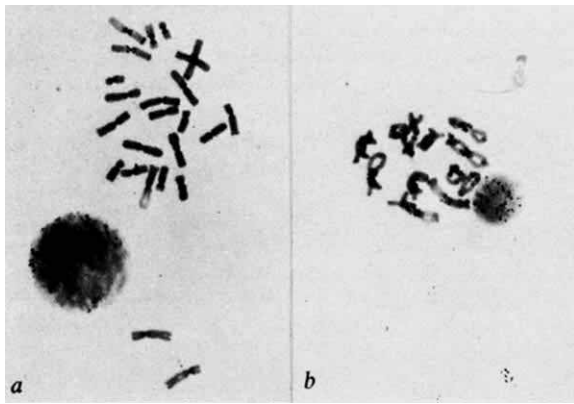


Fig. 1 Spermatogonial metaphase (a) and diakinesis (b) of *T. alpestris* showing C bands.

however, occupied the anterior pole, which later protrudes, developing a close spatial relationship to the acrosome. Of more than 500 spermatid nuclei at this stage, 67.9% had this clearly recognisable asymmetry. We have designated this isolated particule of heterochromatin the acrosomal chromocentre. This asymmetrical distribution of the chromocentres in spermatid nuclei at this stage was evaluated quantitatively using 200 well polarised nuclei. For this purpose the nuclear area was divided into two parts, defined by a line drawn at right angles to the diameter which crosses the acrosomal chromocentre (Fig. 2a). There was an average of 11.31 large chromocentres per spermatid nucleus. This corresponds well with the haploid number of chromosomes ($n=12$) and shows that extensive fusion of chromocentres does not occur at that stage of spermiogenesis.

The average number of large chromocentres in the anterior and posterior areas of the spermatid nucleus was 1.94 and 9.37, respectively. The distribution of the dot-like small chromocentres followed a less pronounced but similar pattern.

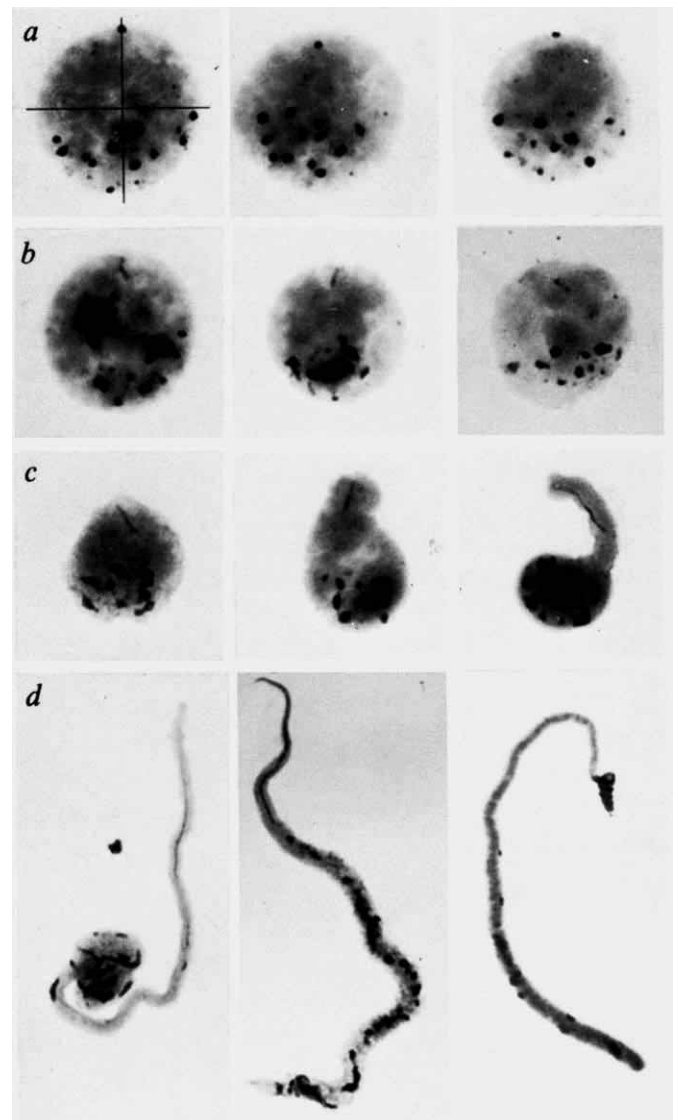
Shortly before the stage at which the spermatid nucleus becomes asymmetrical by the formation of a protrusion at the anterior pole, the acrosomal chromocentre started to elongate and formed a rod-like structure (Fig. 2b). This extended further, concomitant with the growth of the anterior pole, and became a thread (Fig. 2c) which, because of its typical position at the end of the mature sperm nucleus, can be designated the acrosomal chromonema. Very often, a small knob remained visible in the middle of the thread, and in the best preparations a fine beaded structure could be recognised, especially along the anterior segment (Fig. 2d). A homologous structure was detected in mature sperm nuclei of *T. alpestris*, *T. cristatus*, *T. marmoratus* and *D. viridescens*. Demonstration of the acrosomal chromonema in mature sperm nuclei requires relaxation of their tightly spiralised distal part, which can be achieved by brief treatment with Ca^{2+} and by heating the slides. With the DNA-specific bis-benzimidazol stain (Hoechst 33258), the whole sperm nucleus fluoresced brightly, preventing demonstration of the acrosomal chromonema. After mild treatment with deoxyribonuclease, however, this structure became clearly visible as a brightly fluorescent thread (Fig. 3).

None of the somatic tissues examined showed the peculiar arrangement of chromocentres found in spermatids. Nuclei of cells from liver, kidney, intestine, bone marrow and oviduct have randomly distributed chromocentres after C banding. Thus, the polarised arrangement of chromocentres is specific for the postmeiotic cells during spermiogenesis.

To which chromosomes does the acrosomal chromocentre belong? As well as the segment of pericentric hetero-

chromatin, *T. vulgaris* has a heterochromatic region at the distal segment of the long arm of chromosome 1. This region was discovered by Nardi *et al.*⁶ in premeiotic metaphases and can also be recognised on the largest bivalent in Fig. 1. If the acrosomal chromocentre is formed by this block of distal heterochromatin, Macgregor and Walker's description of U-shaped chromosomes with posterior centromeres would still apply to all centromeres. If the plethodontid salamanders they investigated also have a distal block of heterochromatin forming an acrosomal chromocentre, this would probably not be detectable by their method of *in situ* hybridisation with RNA homologous to the minor component DNA. It is likely that the distal C band is built up by a different kind of satellite DNA, representing a very small part of the total DNA. An alternative model would imply that one of the centromeres migrates to the anterior pole shortly before acrosome formation. New differential staining methods (especially with fluorescent dyes) could help to identify the acrosomal chromocentre with one of the C bands in the chromosome complements of the *Triturus* species we examined. There

Fig. 2 a, Asymmetrical arrangement of chromocentres in spermatids of *T. vulgaris*. b, First stage of elongation of the acrosomal chromocentre (*T. vulgaris*). c, Growth of the anterior pole and further elongation of the acrosomal chromonema (*T. vulgaris*). d, Very late spermatid (left) and mature sperm heads of *T. marmoratus* (centre) relaxed by Ca^{2+} treatment, and of *T. vulgaris* (right) not relaxed.



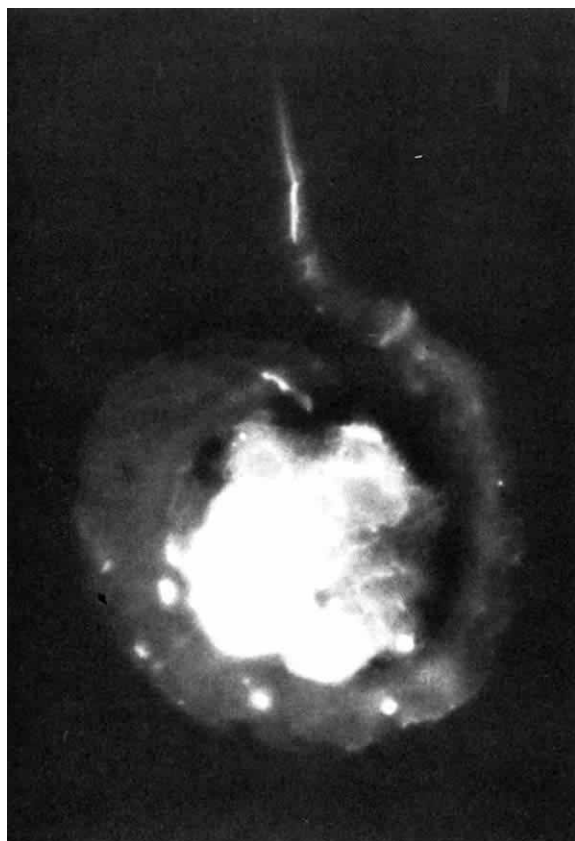


Fig. 3 Late spermatid of *D. viridescens* stained with Hoechst 33258 after mild treatment with deoxyribonuclease. The acrosomal chromonema is clearly visible as a brightly fluorescent thread in the anterior part of developing sperm head.

could be a functional correlation between the acrosomal chromocentre, its morphological alterations during the final stages of spermiogenesis and the location of the genetic information necessary for the organisation of the acrosome. This correlation is also suggested by the presence of peculiar intranuclear and extranuclear structures in differentiating spermatids of a wide variety of species. These structures are found at the part of the nuclear membrane which is first in close contact with the proacrosome granule and later with the growing acrosome⁷.

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M. SCHMID
W. KRONE

Abteilung Humangenetik,
University of Ulm,
79 Ulm (Donau), Oberer Eselsberg, FDR

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Antibody-forming cells in human colostrum after oral immunisation

It has been suggested that feeding infants with human milk reduces the incidence of gastrointestinal infections¹. Although macrophages, lymphocytes and antibodies in the milk are probably important in this protection^{2,3}, the mechanism for the development of specific immunity to enteric pathogens is not known. Human colostrum cells synthesise

IgA (ref. 4), and we have used the haemolysis-in-gel technique to demonstrate that human colostrum contains numerous cells which produce IgA antibodies against the O antigens of commonly encountered *Escherichia coli* bacteria⁵. This suggested that the sensitised lymphocytes had either undergone antigen-induced clonal proliferation within the mammary gland or had homed to that organ from another site after becoming sensitised. Because of reports that gastrointestinal immunisation of germ-free mice led to local⁶ and systemic⁷ production of IgA antibodies, and that cells from Peyer's patches effectively repopulate the ileum of lethally-irradiated rabbits with IgA-producing cells⁸, we have examined the effect of gastrointestinal immunisation on the development of antibody-producing cells in colostrum of humans. We found that oral administration of a non-pathogenic strain of *E. coli* led to the rapid appearance of colostrum cells producing antibodies against the O antigen of the organism.

Three women from our research group consented to ingest 10^9 live *E. coli* 083 bacteria during the last month of their pregnancies. This organism had been used previously to colonise non-pregnant adults (R.M.G. *et al.*, unpublished) and many infants in the first day of life^{9,10} without harmful effects. Therefore, we felt confident that this procedure carried little risk for either the woman or the foetus. The procedure was explained to each subject and verbal consent was obtained. Immediately before ingestion, each subject took 2 g NaHCO₃ to reduce gastric acidity. Before colonisation, samples of serum, parotid fluid and colostrum were collected for the quantitation of antibodies by an enzyme-linked immunosorbent assay^{5,11}, and the *E. coli* in a stool specimen were typed with respect to the O antigen¹². In addition, colostrum was examined for lymphocytes producing antibodies to the lipopolysaccharide (LPS) from the 083 bacteria or a pool of eight more prevalent types of *E. coli* by a modification of the haemolysis-in-gel techniques^{5,13}. Each test was repeated every 3–6 d until the first week *post partum*. In two subjects, a second dose of the bacteria was administered 7 and 10 d after the first.

As previously reported⁵, when a pool of LPS derived from commonly encountered *E. coli* was used as antigen, large quantities of secretory IgA (SIgA) antibodies and numerous IgA-producing cells were found in colostrum (Fig. 1). No consistent change in these values could be related to gastrointestinal immunisation with the *E. coli* of unrelated O type.

Before ingestion of the bacteria, low levels of antibodies to the LPS of *E. coli* 083 were detected in the serum, saliva and colostrum (Fig. 1). Antibodies of the IgA class predominated in the saliva and colostrum and these seemed to be of the SIgA type since the quantitations using an anti- α chain and anti-secretory component (SC) as the enzyme carrier correlated significantly ($r = 0.561$; $P < 0.001$). *E. coli* 083 bacteria, however, were not identified in the stool and cells forming antibodies to the 083 antigen were not detected in the colostrum by the plaque assay before immunisation.

As in infants⁹ and non-pregnant adults (R.M.G. *et al.*, unpublished), ingestion of the *E. coli* 083 did not give rise to symptoms. Furthermore, colonisation with the bacteria was short lived and in none of the individuals did this bacterium dominate the aerobic faecal flora. Most frequently the 083 bacteria could only be identified by serotyping those colonies which were selected by their haemolytic capacity.

Nonetheless, beginning as early as 3 d after the ingestion of the bacteria, cells producing antibodies against the O antigen of the *E. coli* 083 bacteria were detectable in the colostrum from each of the colonised individuals. These cells formed plaques in the presence of developing antisera against α chains or SC, suggesting that the antibodies released were SIgA. The specificity of the plaques produced

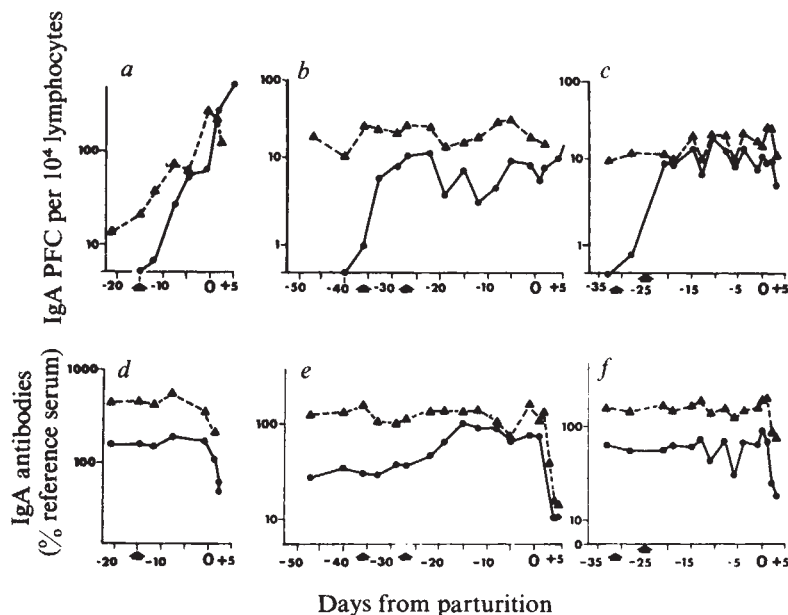


Fig. 1 *a-c*, Effect of gastrointestinal colonisation with *E. coli* 083 on the number of colostral cells producing IgA antibodies against O antigen of immunising bacteria (—) or a pool of commonly encountered *E. coli* O antigens (-----). Human red blood cells (RBC) coated with O antigens or uncoated were mixed with colostral leukocytes in an agar-containing medium. After 1 h incubation (37 °C), anti-immunoglobulin reagents, anti-IgG, anti-IgA or anti-SC (Dakopatts, Copenhagen), were added. Incubation was continued for 1 h, guinea pig complement added for 45 min and plaques counted under oblique light⁵. Significant numbers of plaques were only noted with coated RBC and anti-IgA or SC-developing sera. *d-f*, Quantity of colostral IgA antibodies against the same O antigens. Alkaline phosphatase conjugated to the same anti-immunoglobulin reagent was used in the enzyme-linked immunoabsorbent assay⁵. The results are expressed as percentage of the activity of a reference serum. (*a,d*) (*b,e*) (*c,f*) are single individuals; arrows, the time of ingestion of bacteria; each point is the mean of duplicate determinations.

by colostral cells was verified by inhibition with an excess of purified 083 LPS (250 $\mu\text{g ml}^{-1}$) or by inhibiting protein synthesis with puromycin (10 $\mu\text{g ml}^{-1}$). In one individual, the proportion of lymphoid cells forming IgA plaques against *E. coli* 083 increased logarithmically during the 20 d after a single antigen ingestion. At this point, the number of cells forming plaques against this antigen was greater than that against the pool of eight commonly encountered *E. coli* types (Fig. 1*a*). In the two individuals who received two doses of the bacteria, the peak number of cells producing antibody against the O antigen of the immunising bacteria was reached after 14 d. The number of anti-083-producing cells in their colostrum approached that of the cells producing antibody against the pooled antigens, and was maintained throughout the remainder of the observation period (Fig. 1*b* and *c*).

An increase in the level of IgA antibodies to the 083 bacteria was detected in the colostrum by the enzyme-linked immunosorbent assay after the second dose of bacteria in only one of three immunised individuals (Fig. 1*b*). In contrast, 083 plaques of the IgM and IgG classes never increased significantly above the background level noted in assays with uncoated erythrocytes and the levels of IgG or IgM colostral antibodies did not change after immunisation. Further, no increase in salivary or serum antibody of any class was noted.

The disparity between the number of IgA antibody-producing cells and the antibody content of the colostrum, also noted before³, is not explained by the experiments reported here. The finding, however, provides further evidence for *de novo* synthesis of IgA antibodies by colostral cells.

Two possible mechanisms might be considered to explain the prevalence of colostral cells producing type-specific IgA antibodies to *E. coli* present in the gastrointestinal tract. (1) The bacteria or their LPS antigens may enter the blood from the gastrointestinal tract and cause a clonal proliferation of the antibody-producing cells or their precursors in the mammary tissue. This seems unlikely because of the mild degree of colonisation and the lack of serum antibody response to the administered organisms. (2) The immunological specificity might be transferred from the gastrointestinal tract to the breast by sensitised cells. Because of the lack of a systemic immune response to the ingested antigens, we favour this possibility. Our experiments do not indicate whether these hypothetical transferred cells are the same as those forming haemolytic plaques or whether they are cells which collaborate in the immune response.

Our observations are supported by the report of SIgA

antibodies in the milk of rabbits fed DNP-bovine γ globulin and DNP-type III pneumococcal vaccine¹⁴, and the occurrence of IgA antibodies against *Salmonella typhimurium* in milk of three women infected with this bacteria during pregnancy¹⁵. Because only free antibodies in the milk were measured in those studies, however, the possibility of transfer of antibody from the serum could not be eliminated.

Our studies indicate a close relationship between antigenic sensitisation by way of the gastrointestinal tract and the development of SIgA antibody-producing cells in the milk. We propose that this phenomenon is due to a selective transfer of sensitised lymphocytes from the gastrointestinal tract to the mammary gland. Since the mother is exposed to the microorganisms which the newborn is likely to encounter, the passive immunity provided by this mechanism could be important in the protection of the infant's gastrointestinal tract.

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R. M. GOLDBLUM*
S. AHLSTEDT
B. CARLSSON
L. Å. HANSON
U. JODAL
G. LIDIN-JANSON
A. SOHL-ÅKERLUND

Department of Immunology,
Institute of Medical Microbiology,
Department of Pediatrics,
University of Göteborg,
Göteborg, Sweden

Received May 12; accepted September 12, 1975.

* Present address: Division of Immunology and Allergy, Department of Pediatrics, The University of Texas Medical Branch, Galveston, Texas 77550.

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Separation of antiviral activity of human interferon from cell growth inhibitory effect

It is well established that potent interferon preparations besides inducing antiviral activity¹, inhibit growth of homologous cells in culture²⁻⁴. In addition, interferon increases the susceptibility of cells to the toxic effects of synthetic double-stranded RNA⁵ and to sensitised lymphocytes⁶, enhances phagocytic activity⁷ and stimulates interferon production by priming⁸. In most studies a close correlation was found between antiviral and non-antiviral activities. The correlation is independent of the degree of purification, and efforts at separation have largely been unsuccessful^{2,5,8,9}, indicating that the effects are attributable to the same substance.

Contradictory findings have also been reported. Some interferon preparations were apparently enriched in one of the effects¹⁰, others contained factors antagonistic to the non-antiviral activities³. A partial separation of effects has also been described¹¹. All these experiments were done with the mouse interferon system. The few data obtained on human interferon^{4,12-14}, although comparable with those found with mouse interferon, are insufficient to characterise the system. Although physico-chemical differences have been described, in principle interferons from different species are believed to have an identical mode of action. Recently initiated clinical trials have accentuated the need for a closer examination of the human system. The results reported in this study indicate that it is possible to separate the growth inhibitory effect of human interferon from the antiviral activity by a method based on the selective adsorption of human interferon to albumin. Human interferon was obtained from the supernatant of Sendai virus-induced leukocytes¹⁵. Antiviral activity was assayed by the infectivity inhibition microtest¹⁶. Titres were adjusted to the international standard preparation, 69/19. The preparations were simultaneously assayed for their effect on cell growth. Aliquots of 10⁵ human embryo lung cells (HEL) were seeded into at least five tubes per sample with medium containing interferon, and

Table 1 Growth inhibitory effect promoted by 50 U interferon per ml from different steps during purification of human leukocyte interferon

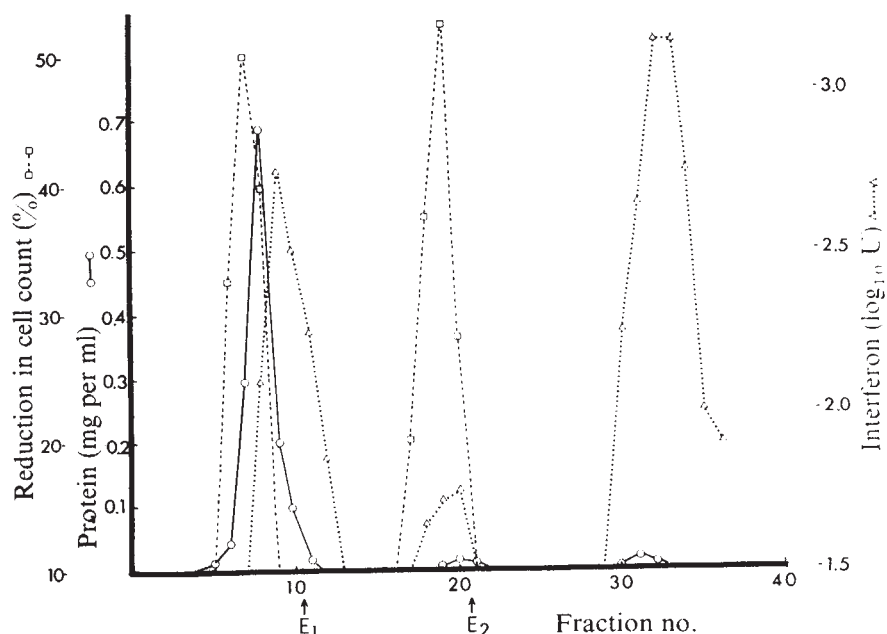
Purification step	Interferon (U per mg protein)	Reduction in cell count (%)
Crude interferon	0.6×10^3	24
Alcoholic precipitate at pH 4.5	2.0×10^3	22.5
Alcoholic precipitate at pH 5.7	3.0×10^3	23
Alcoholic precipitate at pH 8.1	6.0×10^3	24
Partially purified (Cantell)	7.0×10^3	28

Interferon was induced in human leukocytes by Sendai virus¹⁵. Crude interferon preparations were precipitated with 0.5 M KSCN, the precipitate dissolved in acid alcohol and purified by stepwise elevation of the pH. The partially purified (Cantell) preparation was produced in the same way by Dr K. Cantell, University of Helsinki, Finland. 10⁵ HEL cells per tube were seeded in Eagle's MEM with 2% calf serum. After 3 d of incubation at 37 °C the tubes were treated with trypsin-versene and counted in a haemocytometer. Control tubes without interferon represent 100%.

into control tubes without interferon. Cultures were incubated at 37 °C in a 5% CO₂ atmosphere. On the basis of preliminary studies indicating a straight line dose dependence, 50 U interferon per ml were routinely used. For growth curves at least five tubes were counted every day after trypsin-versene treatment. In a typical experiment 78×10^3 , 130×10^3 , and 244×10^3 cells per tube were found for the controls at day 1, 2, and 3 respectively against 88×10^3 , 111×10^3 , and 162×10^3 respectively for the interferon-treated cells. The standard deviation never exceeded 10%. By further incubation the difference between interferon-treated cells and controls levelled off as the control cells entered the stationary phase. On the basis of these results 3 d of incubation were routinely used in tests for cell growth inhibition.

The consecutive steps of purification of the crude interferon preparations, including a highly purified sample from Dr K. Cantell¹⁵ were tested for antiviral and cell growth inhibitory activities (Table 1). The effect on cell growth was strictly correlated to the antiviral effect, regardless of the degree of purification, in accordance with earlier findings^{2,3,5,9}. Interferon preparations were applied to an albumin-Agarose column¹⁷ (Fig. 1). At low ionic strength (phosphate-buffered saline (PBS) (0.15 M NaCl) pH 7.4) the bulk of contaminating proteins could be washed off the column together with unattached interferon. The major part of interferon activity was bound to the column. The eluant was then changed to PBS (1 M NaCl)

Fig. 1 Affinity chromatography of human leukocyte interferon on albumin-coupled Agarose column. CNBr activated Sepharose 4B was swollen in dilute HCl and coupled with bovine albumin by mixing for 2 h. 9 mg of albumin were bound to 1 ml of Agarose beads. Crude interferon, 2×10^4 U in 2 ml, was applied to a PBS equilibrated column. The column was first washed with phosphate-buffered 0.15 M NaCl at pH 7.4. The eluant was changed after the tenth fraction (5 ml each) to PBS (1 M NaCl) at pH 7.4, and after the twentieth fraction to 33% ethylene glycol in PBS (1 M NaCl). All fractions were tested for protein content by the method of Lowry²⁰, antiviral activity, and effect on cell multiplication.



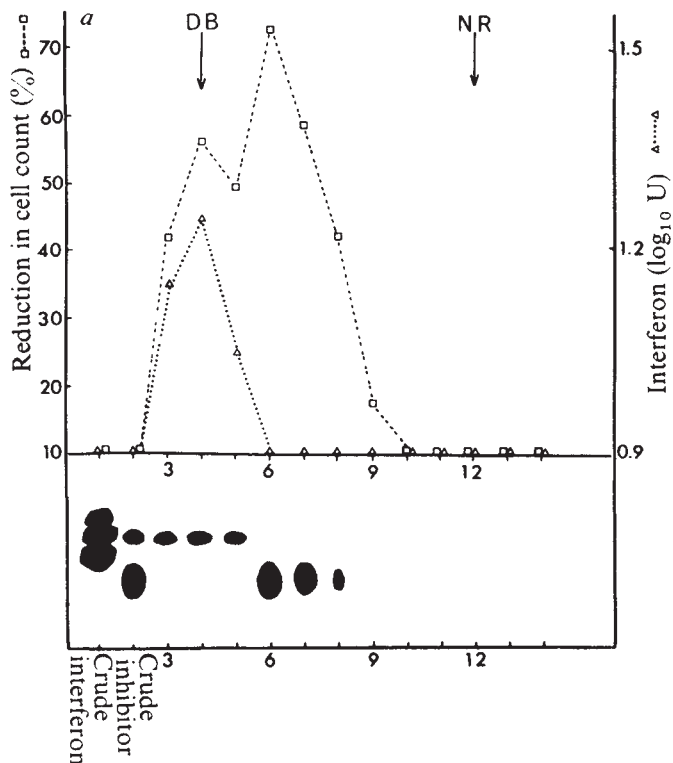


Fig. 2 a, Separation of antiviral activity from the effect on cell growth on a Sephadex G-25 column. The fractions from the affinity chromatography, containing cell growth inhibitory effect (Fig. 1, fractions 17-20), were pooled, concentrated by lyophilisation and applied to a Sephadex G-25 column. Each fraction (5 ml) was tested for antiviral activity and for effect on growth of HEL cells in 1:20 dilution. The arrows indicate the appearance of the included markers: DB, Dextran blue, molecular weight 150,000; NR, neutral red, molecular weight 1,000. Thin-layer chromatography of the fractions on Eastman cellulose sheets, developed in alcohol-acetic acid-4% NaOH (50:20:30) and detected in ninhydrin reagents, is shown in the lower part of the figure. Crude interferon and concentrated pool of the fractions containing cell growth inhibitory effect are included as controls. **b**, Analysis of the fractions from the affinity chromatography, containing antiviral activity (Fig. 1, fractions 30-34) on a Sephadex G-25 column. Thin-layer chromatography of the fractions is shown on the lower part of the figure. Preparation and analysis of the fractions as for **a**.

at pH 7.4, which eluted a sharp peak of cell growth inhibitory activity with only a small antiviral effect. The remaining antiviral activity was eluted in a single peak with 33% ethylene glycol in phosphate-buffered 1 M NaCl (v/v) at pH 7.4. These fractions had no effect on cell growth in dilutions containing 100 U interferon, expected to depress the cell growth by 30-40%. Nor was there any detectable effect on the growth of HeLa cells, which were more sensitive than HEL to the effect of unseparated preparations. Control preparations obtained from the supernatant of uninduced leukocytes and chromatographed as the interferon preparations, showed neither antiviral activity against VSV nor effect on the growth of HEL or HeLa cells.

The fractions showing growth inhibitory effect were examined further. Dialysis against PBS eliminated the growth inhibitory effect, suggesting a low molecular substance, distinct from the interferon molecule with reported molecular weight ~20,000 (refs 18 and 19). Pooled fractions, concentrated by lyophilisation, were applied to a Sephadex G-25 column (Fig. 2a). Antiviral and some growth inhibitory effect were eluted by PBS in the void volume and resulted in a single spot in the thin-layer chromatogram (Fig. 2a, lower part). Growth inhibitory effect without detectable antiviral activity was eluted at molecular weight ~3,200, and these fractions gave rise to single spots in the chromatogram, but with a different retention factor, indicating the separation of the two effects in distinct molecular components. The antiviral fractions obtained by the affinity chromatography were treated by the same process (Fig. 2b). The antiviral activity was eluted in the void volume, and no cell growth inhibition could be detected in any of the fractions. The results were confirmed by thin-layer chromatography: the antiviral fractions resulted in spots with retention factors distinct from those fractions where both activities were detected.

Dialysis of crude human leukocyte interferon against 1 M NaCl resulted in the reduction of the growth inhibition to less than half the normal activity. When the interferon was unfolded by treatment with 5 M urea before dialysis against 1 M NaCl, however, the growth inhibitory activity was completely lost. This result may suggest that the growth inhibitory component is situated inside the interferon molecule and may be released only by forces, which alter the tertiary structure.

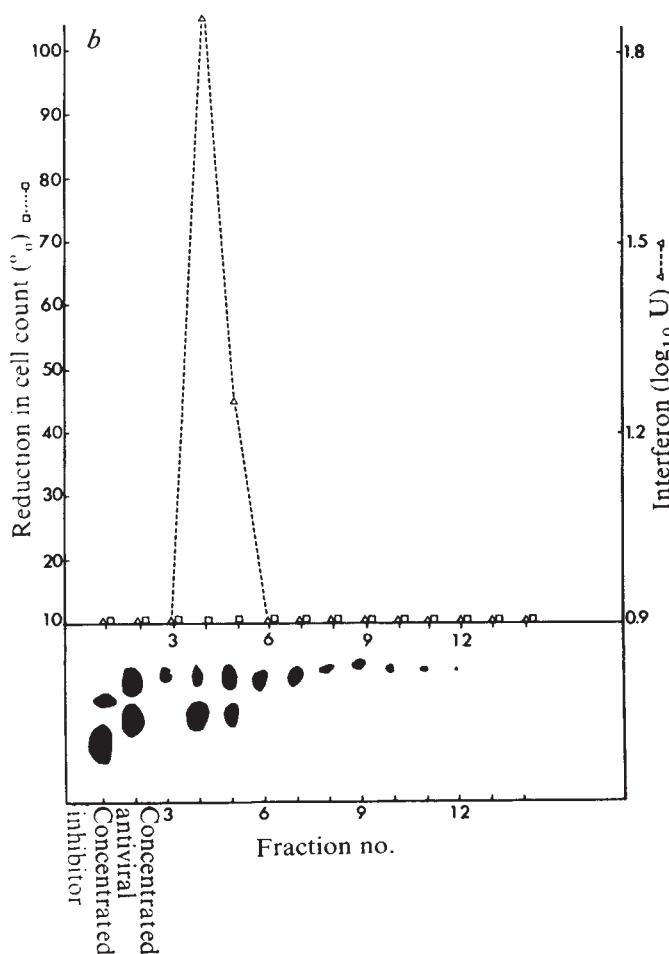
The results reported here indicate that the antiviral activity of interferon can be separated from the effect on cell growth by a relatively simple procedure. The separation is probably caused by the high ionic strength indicating that the molecules responsible for the two different effects are bound to each other by electrostatic bonds.

HELEN DAHL
MIKLOS DEGRE

Kaptein W. Wilhelmsen og Frues,
Bakteriologiske Institutt,
Rikshospitalet,
Oslo 1, Norway

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Specific afferent interference by antiserum of *in vivo* immunity

ANTISERUM can potentially interfere with effective immunity at three different parts of the immune response: afferent, central and efferent¹⁻⁶. Coating or masking of antigenic sites on tumour cells with antiserum could render the cells non-immunogenic^{1,2}—called afferent block—or protect them from attack by immune lymphocytes^{3,4}—called efferent block. In central interference, the ability of the lymphocytes to become sensitised against tumour antigens is suppressed, or immune cells are made non-reactive by the humoral factors^{5,6}. Most early studies in this area were performed with tumour allografts or used antisera with antigenic specificities which were not well characterised. Only a few studies have been performed with syngeneic tumours^{7,8}. Because of the complexity of surface antigens expressed on tumour cells, the effects of the antisera on the tumour-associated transplantation antigen (TATA) was therefore difficult to assess.

We have studied the biological effect of well characterised antisera on the induction of *in vivo* immunity to syngeneic tumours. Three such sera were used. (1) Anti-Friend virus-induced leukaemia serum (AFLS), produced by syngeneic immunisation of C57BL/6 mice with FBL-3 cells, a Friend virus-induced leukaemia. This serum recognised three antigenic specificities: Friend type specific, FMR (Friend, Moloney, Rauscher) and cross-reactive foetal antigens⁹. (2) Anti-SV40 tumour serum (ASVS) produced by immunisation of (BALB/c × C57BL/6)F₁ with a syngeneic SV40 tumour. This serum would react chiefly with a SV40 tumour-associated cell surface antigen (TASA)¹⁰. (3) Anti-foetal serum (AFS), produced by immunisation of C57BL/6 mice with syngeneic foetuses. This serum only recognised the foetal antigens expressed on foetal cells or various tumour cells¹¹.

The FBL-3 cells have been shown to be very tumorigenic (intraperitoneal inoculation of 1×10^1 cells would produce progressive tumour growth), and it was also very immunogenic and immunosensitive (immunisation with 1×10^4 X-irradiated cells could produce protection against intraperitoneal inoculation of 1×10^6 FBL-3 cells)¹², and therefore, the FBL-3 cells were very suitable for tests of *in vivo* tumour immunity. In our study, sera of various sources were preincubated with 10,000 r. X-irradiated FBL-3 cells. Serum-treated and untreated cells were then inoculated into B6 mice to determine the effect of serum treatment on the ability of these cells to produce *in vivo* tumour immunity. The results are summarised in Table 1. In experiment 1, 1×10^4 or more untreated irradiated FBL-3 were needed to produce significant protection against challenge with FBL-3 cells. In contrast, 1×10^4 serum-treated cells produced considerably less protection. This blocking of the immuno-

Table 1 Blocking of *in vivo* tumour immunity against FBL-3 cells

Treatment*	Immunisation No. of cells†	Tumour take after challenge (%)‡	% Protec-tion	% Block-ing‡§
Experiment 1				
—	1×10^3	13/15 (87)	13	—
AFLS	1×10^3	11/15 (73)	27	—108
—	1×10^4	5/30 (17)	83	—
AFLS	1×10^4	25/34 (74) ¶	26 ¶	69 ¶
—	1×10^5	1/23 (04)	96	—
AFLS	1×10^5	8/35 (23)	77	20
—	—	10/10 (100)	—	—
Experiment 2				
—	5×10^4	1/17 (06)	94	—
NMS	5×10^4	2/20 (10)	90	4
AFLS	5×10^4	8/15 (53) ¶	47 ¶	50 ¶
—	—	10/10 (100)	—	—
Experiment 3				
NMS	3×10^4	4/16 (25)	75	—
ASVS	3×10^4	6/17 (35)	65	13
AFS	3×10^4	8/19 (42)	58	23
AFLS	3×10^4	14/19 (74) ¶	26 ¶	65 ¶
—	—	19/19 (100)	—	—

Experiments 1, 2 and 3 were separate, individual experiments. NMS, normal mouse serum. In experiments 2 and 3, 0.4 ml undiluted serum was incubated with 1×10^6 10,000 r. X-irradiated FBL-3 cells at 37 °C for 60 min.

* In experiment 1, 0.2 ml undiluted serum was incubated with 5×10^4 , 5×10^5 or 5×10^6 10,000 r. X-irradiated FBL-3 cells, respectively, at 37 °C for 60 min. After treatment, they were resuspended to 10 ml in Hanks' balanced salt solution, and 0.2 ml of each preparation was inoculated intraperitoneally into each mouse (the mice received 1×10^3 , 1×10^4 and 1×10^5 cells, respectively). Untreated X-irradiated FBL-3 cells were given in the same manner.

† Inoculated intraperitoneally with indicated number of 10,000 r. X-irradiated cells.

‡ Mice were challenged intraperitoneally with viable FBL-3 cells. The challenge doses were 1×10^4 – 1×10^6 in experiment 1, 3×10^5 – 3×10^6 in experiment 2, and 3×10^6 cells in experiment 3. The denominator indicates total number of mice challenged, the numerator indicates the number of mice with tumour growth. The figures in parentheses indicate the percentage of mice with tumours. Mice were observed for tumour incidence for 30–60 d. All control mice died within 14–20 d of challenge.

§ (% Protection obtained with) – (% Protection obtained with) untreated cells treated cells $\times 100$

% Protection obtained with untreated cells

¶ $P \leq 0.01$ by χ^2 test, by comparing the group inoculated with antiserum treated cells with the group inoculated with untreated cells (experiments 1 and 2), or with the group inoculated with normal serum-treated cells (experiment 3). In experiment 1, comparisons were made between groups receiving the same number of cells.

genicity by the antiserum was overcome by immunising with 1×10^5 treated cells. In experiment 2, blocking of immunogenicity was again shown to occur with 5×10^4 cells treated with AFLS, but not with cells treated with normal serum. The specificity of the blocking of anti-tumour protection was further shown in experiment 3. Only AFLS produced a significant interference with the immunisation. Sera produced by immunisation with SV40 (ASVS) or by foetal tissue (AFS) had no significant effect.

To determine whether the blocking effect by AFLS could be explained by toxicity to FBL-3 cells, the following experiments were performed. First, FBL-3 cells treated with AFLS were shown to retain viability *in vitro*, as measured by Trypan blue dye exclusion. Second, normal or immune C57BL/6 mice were challenged intraperitoneally with untreated or serum-treated viable FBL-3. The results are summarised in Table 2. Serum treatment had no effect on unimmunised mice, which indicated that AFLS did not cause significant killing of the FBL-3 cells *in vivo*. Such serum treatment, however, increased the protection in immune mice. The mechanism of this potentiation of established *in vivo* immunity was not clear. It may be due to some augmented effect exerted by antiserum on the effector cells; or the presence of unblocking antibodies in

Table 2 Direct effect of antiserum on growth of FBL-3 cells in normal and immune hosts

Immunisation*	Treatment of challenge cells†	Tumour take‡
—	—	14/14 (100)
—	AFLS	14/14 (100)
FBL-3	—	8/18 (44)§
FBL-3	NMS	8/17 (47)§
FBL-3	AFLS	2/18 (11)§ ¶

* Inoculated intraperitoneally with 5×10^4 , 10,000 r. X-irradiated FBL-3 cells.

† 1×10^6 viable FBL-3 cells were incubated with 0.4 ml of undiluted serum at 37 °C for 60 min.

‡ Same as Table 1.

§ $P \leq 0.05$ by χ^2 test, by comparing with unimmunised mice challenged with untreated FBL-3 cells.

¶ $P \leq 0.05$ by χ^2 test, by comparing with immunised mice challenged with untreated FBL-3 cells.

this serum to offset the blocking antibody which might be present in these immune hosts^{13,14}. It seemed to be different from the antibody-dependent, cell-mediated cytotoxicity^{15,16}, however, in which specific cytotoxicity could be produced by the interaction of non-sensitised lymphoid cells and specific antiserum.

Our results indicated that specific antiserum could produce afferent interference with *in vivo* tumour immunity by masking the specific antigenic sites and thereby block the immunogenicity of the tumour cells. This effect was not likely to be due to central interference, since inoculation of 1×10^5 serum-treated cells, which involves administration of at least the same amount of antiserum on the coated tumour cells, had no significant blocking effect (Table 1). These results are more consistent with insufficient masking of the larger number of tumour cells by the amount of available antibodies. This finding also indicated that the blocking was not due to an effect on the effector cells; if it was due to elution of antiserum from the coated tumour cells to act on the effector cells, then blocking should be obtained with 1×10^5 treated cells. The mechanism of blocking also did not seem to be related to a direct cytotoxic effect on tumour cells, as shown by lack of protection in normal mice challenged with serum-treated cells (Table 2). Moreover, the interference of *in vivo* tumour immunity was antigenically specific. Only the serum which contained antibodies against the appropriate TASA was effective. This result was similar to those obtained by testing the specificity by immunisation with various tumour cells or foetal cells¹², that is, *in vivo* protection was only obtained by immunisation with leukaemias induced by Friend, Moloney or Rauscher viruses (FRM specific), but not by immunisation with foetal tissue or tumour of other origin. These findings of the inability to interfere with antitumour immunity by anti-foetal serum provide another indication that foetal antigens in this system did not function as transplantation protection antigens. These data also supported the previous reports of the serologically detected TASA being a transplantation antigen^{9,12}.

This study has shown that only the specific antitumour antiserum can produce afferent interference of the *in vivo* tumour immunity. Thus, when well characterised, specific antisera are used, it can also help to test the specificity of TASA.

CHOU-CHIK TING
RONALD B. HERBERMAN

Laboratory of Cell Biology and Laboratory of
Immunodiagnosis,
National Cancer Institute,
Bethesda, Maryland 20014

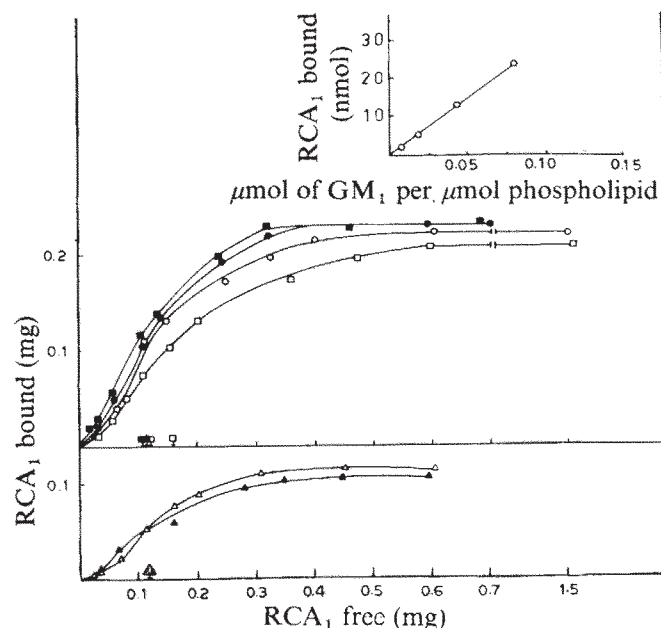
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Interaction between lectin from *Ricinus communis* and liposomes containing gangliosides

A MODEL has been presented showing the contribution of the protein-glycoprotein complex^{1–3} when transformed cells are agglutinated by lectins^{4–6}. But this has not been possible for the lectin-glycolipid complex because glycolipids form micelles, even at very low concentrations. This difficulty could be overcome by incorporating these glycolipids into lipid vesicles. (Haywood demonstrated that ganglioside incorporated in liposomes can provide receptors for sendai virus⁷.) We have now studied the interaction between a galactose-specific lectin from *Ricinus communis* beans^{1,8,9} and liposomes containing different amounts of monosialoganglioside (GM₁), and found that the latter can be used as a model system for the investigation of agglutination of cells by lectins.

Gangliosides GM₁, GM₂, GD_{1a} and GT₁ (according to Svennerholm's nomenclature¹⁰) were isolated by thin-layer chromatography (TLC) from beef brain ganglioside prepared by the method of Folch *et al.*¹¹. Individual gangliosides were more than 90% pure as judged by TLC. *R. communis* lectin was purified as before¹ and lipid vesicles¹² were prepared by Huang's

Fig. 1 Binding of RCA₁ to liposomes of different ratios (*R*) of phospholipid to GM₁ at 4 °C. Various amounts of RCA₁ were added to a liposome suspension containing 6.4 nmol of GM₁ in 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. The total volume of incubation medium was kept to 2 ml. Top, SC liposome: ○, *R* = 123; □, *R* = 55; ●, *R* = 22.8; ■, *R* = 12. Bottom, MC liposome: ▲, *R* = 147; △, *R* = 43.5. Inset, amount of RCA₁ bound as a function of ratio of GM₁ to phospholipid (1/*R*).



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method¹³. Phospholipid (synthetic β - γ -dipalmitoyl-DL- α -lecithin, Sigma) and cholesterol (three times crystallised) in the molar ratio 2:1, with various concentrations of the different gangliosides (0.02–0.1 mol per mol of phospholipid) were dissolved in 2:1 chloroform-methanol. The solvent was subsequently removed under reduced pressure at 25 °C, and the flasks were placed in a vacuum desiccator for 4 h. The lipids were then suspended in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and sonicated for 2 h under nitrogen at 4 °C. The liposomes thus obtained were centrifuged at 5,000*g* for 30 min to remove titanium particles and then subjected to gel filtration on Sepharose 4B to separate single compartment (SC) from multi-compartment (MC) liposomes described by Huang¹³. The fractions of SC and MC liposomes were pooled separately and concentrated to 1 ml. The amount of dipalmitoyl lecithin and ganglioside in the liposome was calculated by estimating lipid phosphorous¹⁴ and *n*-acetylneuraminic acid¹⁵, respectively. About 93% of phospholipid and 90–95% of gangliosides were incorporated in these liposomes. Thus liposomes containing GM₁, GM₂, GT₁ and GD_{1a} had been prepared.

Binding studies were done at 4 °C by adding various concentrations of lectin to the different batches of liposomes containing a constant amount of various glycolipids in 0.05 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl. The tubes were constantly shaken and after 3 h they were centrifuged at 105,000*g* for 2 h. The lectin remaining in the supernatant was estimated for protein by Lowry's method¹⁶ and subtracted from the amount added to give the amount bound to liposomes.

Liposomes without GM₁ and GM₂, GT₁ and GD_{1a} did not bind lectin whereas those containing GM₁ showed considerable binding (Fig. 1). Since all the lectin was recovered in the supernatant in the case of liposomes without GM₁, in the conditions of the binding experiment these way no significant entrainment of lectin. The association constant K_a (mol⁻¹) for the binding of incorporated GM₁ to lectin was calculated according to equation (1) from the value of m at $\theta = 0.5$.

$$K_a = \frac{\theta}{(1-\theta)m} \quad (1)$$

(where m = concentration of RCA₁ free in solution and θ = fraction of GM₁ at the outer surface in liposome, bound to RCA₁).

K_a thus calculated has a value of 2.2×10^6 mol⁻¹. Table 1 shows that about 27% and 60% of total GM₁ in liposome is available to bind to lectin at the outer surface of MC and SC liposomes, respectively. This agrees well with the amount of

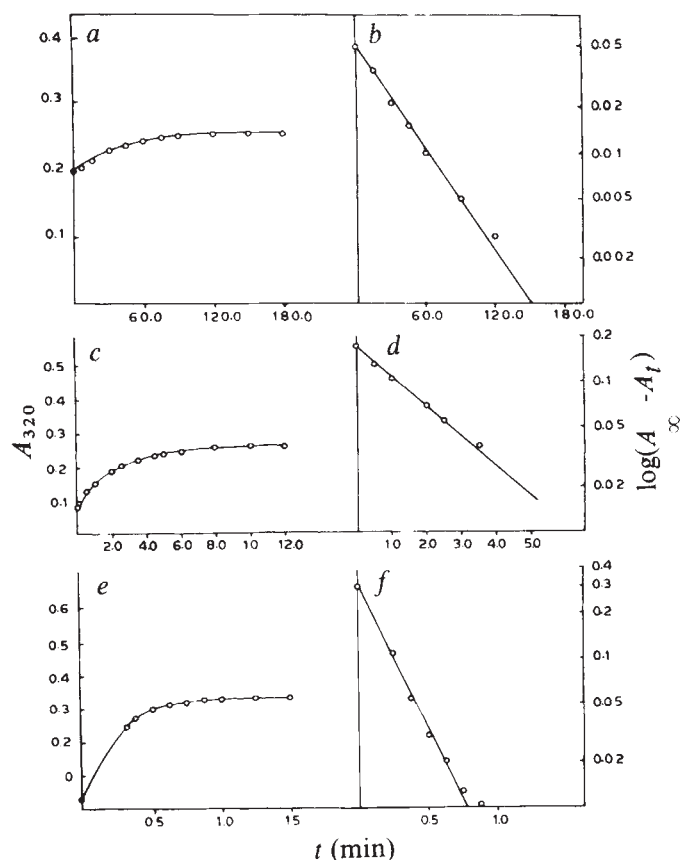


Fig. 2 Typical run for the kinetics of SC liposome aggregation on mixing with RCA₁ at 28 °C. Change in absorption at 320 nm with time on mixing of RCA₁ and liposome containing GM₁ with different R values in 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. *a*, $R = 123$; 3.5×10^{-7} mol of GM₁ in liposome and 2.3×10^{-6} mol of RCA₁. *c*, $R = 55$; 3.4×10^{-7} mol of GM₁ in liposome and 3.04×10^{-6} mol of RCA₁. *e*, $R = 22.8$; 2.37×10^{-7} mol of GM₁ in liposome and 1.66×10^{-6} mol of RCA₁. The corresponding semilog plots of *a*, *c* and *e* are presented in *b*, *d* and *f*, respectively.

n-acetylneuraminic acid liberated from GD_{1a} incorporated in liposome when treated with *Vibrio cholerae* neuraminidase (shown in parentheses in column 5 of Table). Binding of lectin to GM₁ liposome was prevented by lactose. The precipitin reaction between guar gum and lectin was not inhibited by GM₁ either below or above the critical micellar concentration¹⁷,

Table 1 Thermodynamic and kinetic parameters of the interaction between RCA₁ and liposomes with a different ratio (R) of phospholipid to GM₁ in 0.05 M phosphate buffer pH 7.0 containing 0.15 M NaCl

Type of liposome	Composition of liposome* Phospholipid (μ mol ml ⁻¹)	GM ₁ † (μ mol ml ⁻¹)	Amount of RCA ₁ bound (μ g μ mol ⁻¹ of phospholipid)	% of GM ₁ ‡ available for RCA ₁ binding	RCA ₁ binding sites per μ m ² § $\times 10^{-3}$	Association constant (K_a) (4 °C) $\times 10^{-6}$ mol ⁻¹	Rate of formation of clusters (k_t (mol ⁻¹ s ⁻¹) at 28 °C $\times 10^{-3}$)
SC	10.5	0.085 (123)	264.0	55 (65 $\pm$ 5%)	1.65	2.04	0.15 $\pm$ 0.02
SC	10.0	0.185 (55)	635.0	56 (65 $\pm$ 6%)	4.10	1.52	2.3 $\pm$ 0.2
SC	10.8	0.475 (22.8)	1,560.0	60 (—)	10.40	2.18	43.0 $\pm$ 0.3
SC	10.5	0.87 (12)	2,886.0	58 (—)	19.0	2.22	Too fast to measure¶
MC	12.5	0.085 (147)	110.0	25 (—)	—	2.20	0.12 $\pm$ 0.03
MC	12.0	0.275 (43.5)	403.0	27 (30 $\pm$ 3%)	—	2.20	2.4 $\pm$ 0.15

The results presented in columns 5, 6, 7 and 8 are the average of three independent experiments.

*Dipalmitoyl lecithin and ganglioside (GM₁) in liposome was calculated by estimating lipid phosphorous¹⁴ and *n*-acetylneuraminic acid¹⁵, respectively.

†Numbers in parentheses indicate values of R (phospholipid per GM₁).

GM₁ available at the surface was calculated using a saturation value for RCA₁ considering RCA₁ bivalent.

‡This was confirmed by preparing GD_{1a} liposome and subjecting it to *Vibrio cholerae* neuraminidase.

§The number of molecules of *n*-acetylneuraminic acid liberated from these liposomes corresponds to the amounts of GD_{1a} molecules accessible to hydrolysis by neuraminidase (that is exposed on the outer surface of liposome). This number is indicated in parentheses under ‡.

¶Based on the molecular weight of SC liposome as 2×10^6 daltons and its diameter as 300 Å (ref. 13).

¶¶For a 1-ml reaction mixture containing 1.73×10^{-7} mol of GM₁ and 3×10^{-6} mol of RCA₁.

suggesting that there was no binding of free GM₁ with lectin.

When lectin was added to liposome containing GM₁, the absorbance (that is turbidity) at 320 nm increased with time presumably due to the formation of liposome clusters which resemble cell agglutination. The rate at which turbidity increased at 320 nm was dependent on the composition of the liposome, that is on the number of lectin binding sites per μm^2 , although K_a remained constant. Figure 2 shows the typical kinetic run for SC liposomes with different ratios of phospholipid to GM₁ (R values), carried out with lectin in excess GM₁ available at the outer surface (pseudo-first order conditions). From the slope of plot of $\log(A_\infty - A_t)$ against t (where A_t and A_∞ are the absorbance at time t and infinity) the pseudo-first order rate constant k_{obs} is calculated. There from the second-order rate constant of cluster formation, $k_f = (k_{obs}/C)$, where C is the concentration of lectin in mol^{-1} based on a molecular weight of lectin of 120,000) are summarised in Table 1. It is interesting that a factor of two increase in available binding sites for lectin leads to an increase in the rate constant of formation by a factor of 20. The k_f value for GM₁ liposome ($R = 55$) is comparable with that for the glycoprotein-concanavalin A (Con A) system¹, whereas k_f for the liposome ($R = 22.8$) is comparable with that for the 'precipitin' reactions between glycogen and con A¹ and between guar gum and *R. communis* lectin. The latter values compare well with the rate constant of $8 \times 10^4 \text{ mol}^{-1} \text{ s}^{-1}$ for the formation of the soluble con A-carbohydrate complex, reported by Gray and Glew¹⁸.

From our measurements we draw the following conclusions. (1) The incorporated gangliosides are recognised through the non-reducing terminal galactose moiety; this is consistent with our studies with galactose-containing glycoproteins¹⁹. (2) The K_a of this interaction is 100–1,000 times greater than those of galactose and *p*-nitrophenyl- β -D-galactopyranoside, respectively^{1,9}. This value may arise from any of the following and/or any combination of (i) interaction of protein component with the lipid matrix, (ii) different conformations of carbohydrates of the glycolipid when embedded in lipid phase and when free in solution, (iii) alteration of the polarity of the intervening medium due to the presence of the glycolipid cluster. Any differences in these factors may lead to slight differences in K_a values calculated from the 50% saturation values. (3) The rate constant of cluster formation is significantly altered depending on the number of sites per μm^2 of liposome surface which are likely to be uniformly distributed, whether they are MC or SC. This suggests that GM₁ in liposome which are available to bind with lectin with more or less the same affinity are kinetically distinguishable when the ratios of GM₁ and phospholipid in the liposome are varied. The reason for this is not clear. It may be because GM₁ is differently located in the lipid matrix or because surface diffusion controls the rate of formation of clusters, as measured by increased turbidity. Nevertheless our findings are consistent with the idea that the observed increased agglutinability of transformed cells is mediated through (1) change in the topological distribution of receptor site and (2) membrane fluidity. Further studies are required to assess quantitatively how the membrane fluidity (determined by the nature and composition of lipid matrices) influences the kinetic behaviour of cluster formation representing cell agglutination.

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AVADHESHA SUROLIA
B. K. BACHHAWAT

Neurochemistry Laboratory,
Department of Neurological Sciences,
Christian Medical College Hospital,
Vellore-632004

S. K. PODDER

Department of Biochemistry,
Indian Institute of Science,
Bangalore-560012, India

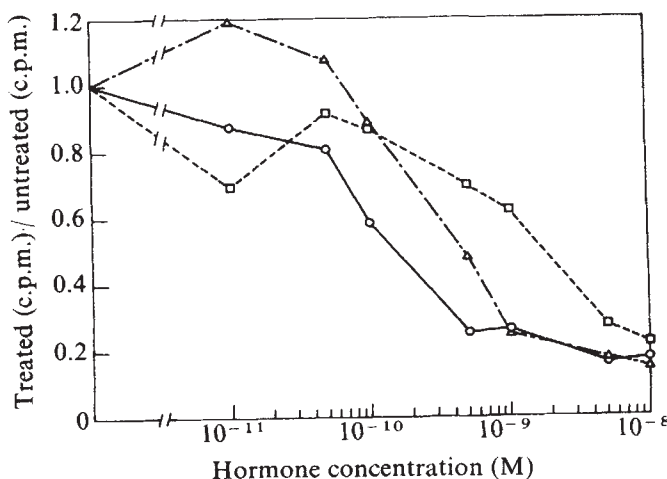
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Differential effects of glucocorticoids on DNA synthesis in normal and virus-transformed chick embryo cells

LOOSE connective tissue fibroblasts in the mouse become markedly non-motile and refractile and lose pinocytotic activity within about an hour of hydrocortisone infusion. Only minutes are required to mimic this response in culture¹. These responses of fibroblasts are well correlated with the topical anti-inflammatory potency of the glucocorticoids². Glucocorticoids inhibit the multiplication in primary cultures of rat and mouse fibroblasts and of human epithelial cells^{3–6}. In marked contrast, they stimulate multiplication of 3T3 cells⁷. Some investigators regard the 3T3 cell as a prototype for the study of growth regulation in

Fig. 1 Inhibition by glucocorticoids of DNA synthesis in cultures of chick embryo cells. 10^6 chick embryo cells were seeded in 60-mm Petri dishes. After 3 d growth, the medium was changed to one without serum, and one of three glucocorticoids was added at the indicated concentrations for 16 h. The rate of ^3H -TdR incorporation into DNA for 1 h was measured by extraction and scintillation counting, and the total protein content was measured¹². $\circ$, Dexamethasone; $\triangle$, hydrocortisone; $\square$, cortisone. We used published cell and virus culturing techniques^{8–11}. DNA synthesis was measured by the incorporation of ^3H -thymidine (^3H -TdR) into extractable DNA, or by autoradiography¹². Protein was determined by the Lowry method for cells in culture¹². Glucose transport was measured by the uptake of ^3H -2-deoxy-D-glucose (^3H -2dGlc)¹². In several preliminary experiments, we observed that glucocorticoids did not affect DNA synthesis in chick embryo cultures if the hormones were added to medium conditioned by one or more days of exposure to growing cells. We therefore carried out all experiments with cultures that received fresh changes of medium.



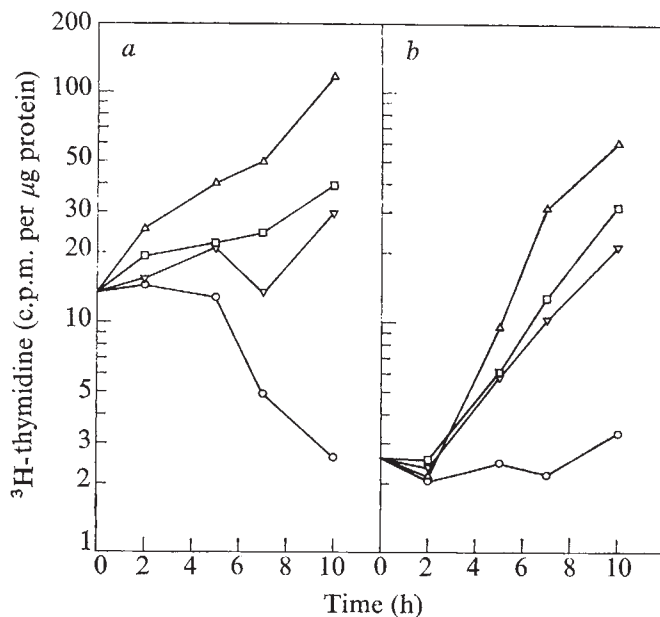


Fig. 2 Kinetics of inhibition and restoration of DNA synthesis in chick embryo cultures after hydrocortisone treatment and removal. Cultures of chick embryo cells were treated for 16 h with serum-free medium (a) or with serum-free medium containing 10^{-7} M hydrocortisone (b). The following media then were substituted in both groups: Δ , 1% serum, no hydrocortisone; $\square$, 1% serum, with hydrocortisone; ∇ , no serum, no hydrocortisone; $\circ$, no serum, with hydrocortisone. At the indicated times, the incorporation for 1 h of ^3H -TdR into DNA was determined.

cultured fibroblasts, although this line has been identified as tumorigenic in some conditions⁸.

We have determined the effects of glucocorticoids on primary chick embryo fibroblast cultures in which a variety of growth-regulatory parameters were measured, and on the same cells after they had undergone virus-induced malignant transformation. We found that the glucocorticoids in very low concentrations retarded the progress of normal chick embryo fibroblasts through the G_1 period of the cell cycle, thereby reducing the fraction of cells synthesising DNA at any given time. The same concentrations of glucocorticoids had no effect on the initiation of DNA synthesis in virus-transformed cultures.

Figure 1 shows the effects of treatment for 16 h with each of three glucocorticoids on DNA synthesis in normal chick embryo cells. (We chose 16 h after hormone treatment as the time for measuring maximum inhibition of DNA synthesis, as shown in Fig. 3.) All three glucocorticoids reduced ^3H -TdR incorporation into DNA about four- to sixfold. Levels of 1.5×10^{-10} M dexamethasone, 4.5×10^{-10} M hydrocortisone and 1.5×10^{-9} M corticosterone caused 50% inhibition of DNA synthesis. These levels are all within the physiological concentration range of glucocorticoids¹³.

We measured the kinetics of inhibition and restoration of DNA synthesis by hydrocortisone in the presence or absence of serum. Inhibition of DNA synthesis definitely was evident both in the presence and absence of serum within 4 h of administration of the hormone, and inhibition became more pronounced with time up to 10 h (Fig. 2a). Similarly, increased DNA synthesis was apparent within 4 h of removal of hydrocortisone and became more pronounced with time up to 10 h (Fig. 2b). Inhibition by the hormone of the rate of incorporation of ^3H -TdR into extractable DNA was proportional to reduction in the fraction of nuclei labelled with ^3H -TdR, as detected by autoradiography (Fig. 3).

The uptake of ^3H -2-dGlc into uninfected cells was inhibited more rapidly than was the incorporation of ^3H -TdR

into DNA, but the extent of inhibition at 16 and 24 h was about the same for both substances (Fig. 4). Hydrocortisone in concentrations as high as 1.0×10^{-8} M had no effect on DNA synthesis in cultures of chick embryo fibroblasts transformed by infection with Rous sarcoma virus (Table 1).

These data indicate that the growth of normal fibroblasts in primary culture is inhibited by glucocorticoids, just as it is in the animal. This result contrasts markedly with the stimulatory effect of glucocorticoids on the growth of the 3T3 cell line⁷, which has been widely accepted as the prototype of normalcy for fibroblasts in culture. The demonstration that 3T3 cells produced haemangioendotheliomas in mice in certain conditions⁸ raises serious questions about the use of 3T3 cells for studying growth-regulatory processes. These doubts are accentuated by the paradoxical response of the cells to glucocorticoids.

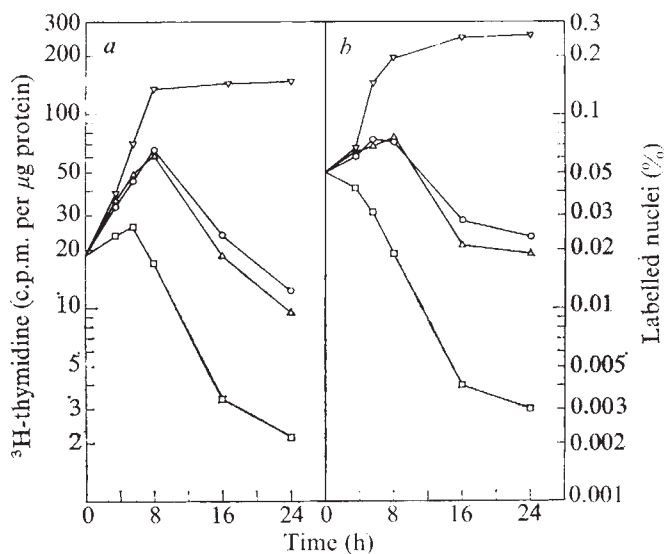


Fig. 3 Comparison of scintillation counting and autoradiography in measuring effects on DNA synthesis with time after hydrocortisone treatment. Cultures of chick embryo cells were treated for various times with 10^{-7} M hydrocortisone in the presence or absence of 1% serum. ^3H -TdR incorporation into DNA was measured by extraction and scintillation counting (a), and by autoradiography (b). ∇ , 1% serum, no hydrocortisone; Δ , 1% serum, with hydrocortisone; $\circ$, no serum, no hydrocortisone; $\square$, no serum, with hydrocortisone.

We have shown that the inhibition of growth by glucocorticoids is associated with a reduction in the number of cells synthesising DNA that is proportional to the reduction in ^3H -TdR incorporation into extractable DNA. This indicates that glucocorticoids have no effect on DNA chain elongation and act by slowing the progress of cells through the G_1 period of the cell cycle. Thus, the effect is similar to that produced by increasing population density or by

Table 1 Comparative effect of hydrocortisone on normal and virus-transformed chick embryo cells

Hydrocortisone (M)	^3H -thymidine Uninfected (c.p.m. per μg protein)*	Transformed
Control	13.54	101.70
10^{-7}	2.95	117.20
10^{-6}	3.01	112.46
10^{-5}	3.05	103.44
10^{-4}	2.75	30.46

*Average of duplicates 16 h after initiation of treatment. Cultures were prepared as described in Fig. 1, and half were infected with the Schmidt-Ruppin strain of Rous sarcoma virus (1×10^8 focus-forming units). The medium was changed on both sets of cultures after 36 h and again on day 3. The latter change of medium contained the indicated hydrocortisone concentrations. ^3H -TdR incorporation into DNA was measured 16 h after hormone treatment.

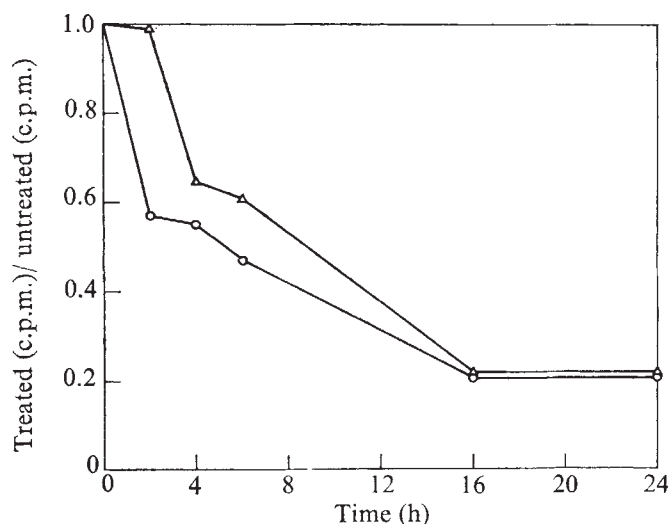


Fig. 4 Kinetics of inhibition by hydrocortisone of $^3\text{H-2-dGlc}$ uptake and $^3\text{H-TdR}$ incorporation. The medium of 3-d-old chick embryo cultures was changed to one without serum, and 10^{-7} M hydrocortisone was added to half the cultures. At the indicated times, measurements were made of $^3\text{H-2-dGlc}$ uptake into acid-soluble material and of the incorporation of $^3\text{H-TdR}$ into acid-insoluble material. Protein content was determined by the Lowry method. $\circ$, $^3\text{H-2-dGlc}$; $\triangle$, $^3\text{H-TdR}$.

lowering either serum concentration or pH (ref. 11). Glucocorticoids produce the same inhibitory effect as these treatments on the uptake of $^3\text{H-2-dGlc}$ and have similar rapid kinetics.

Overall, these results suggest that inhibitory treatments act through a common mechanism related to the hypothetical mechanism by which diverse growth-stimulatory agents act^{14,15}. In the latter hypothesis, the coordinated control of many processes in chick embryo cells is effected through regulation of the availability of divalent cations for enzymatic reactions and other cellular functions¹⁵. The most important of the cations for metabolic control is Mg^{2+} , because of its essential role in transphosphorylation reactions which are the regulatory steps in many biochemical pathways. A mechanism for control of divalent cation activity is suggested by the observation that the stimulatory hormone insulin, decreases the binding of Ca^{2+} to plasma membranes, whereas hydrocortisone increases that binding¹⁶. Substances that modulate Ca^{2+} binding to membranes are also likely to affect Mg^{2+} binding as well, as both cations have similar binding properties for membranes¹⁷. The availability of Mg^{2+} for metabolic processes depends on its partitioning between free and membrane bound forms¹⁷.

Hydrocortisone failed to inhibit DNA synthesis in virus-transformed cultures at a concentration almost 10^5 times higher than that which produces 50% inhibition in normal cells. This observation supports the claim that tumour cells lose their sensitivity to the insulin-anti-insulin set of controls found in normal cells¹⁸. The regulatory mechanism proposed above suggests that membranes of tumour cells have diminished capacity for binding divalent cations and that this characteristic cannot be modified by glucocorticoids. Information on whether this is an intrinsic defect of the membrane or a response conditioned by the internal cell milieu should shed some light on the mechanism of carcinogenesis.

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DOUGLAS W. FODGE*
HARRY RUBIN

Department of Molecular Biology and
Virus Laboratory,
University of California,
Berkeley, California 94720

Received July 14; accepted August 15, 1975.

*Present Address: Medical Sciences Department, Stanford Research Institute, Menlo Park, California 94025.

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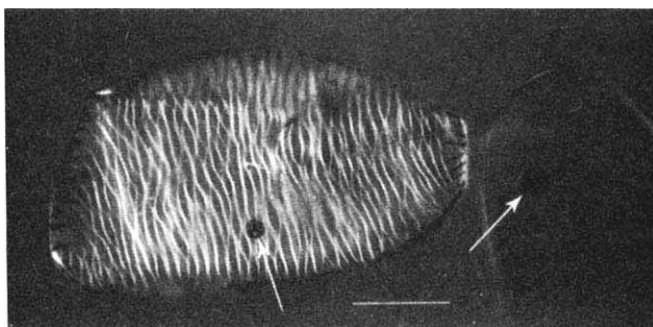
Differentiation of wound vessel members without DNA synthesis, mitosis or cell division

THE formulation of a general model of gene regulation in eukaryotic organisms would be facilitated were it known when the molecular events responsible for reprogramming of genetic information occur during the cell cycle. Evidence from various systems in which animal cells differentiate *in vivo* and *in vitro* suggests that certain characteristics of the fully differentiated state are not manifest until the cells undergo DNA synthesis and nuclear division¹. Similarly the differentiation of wound vessel members (WVM) in plant parenchyma tissue is thought to occur only after cells have replicated at least once in inductive conditions².

WVM are highly specialised elements of the xylem which differentiate in parenchyma tissue as a response to wounding. They are characterised by the deposition of a patterned cellulose secondary wall which becomes lignified and remains as a resistant structure when the cell contents undergo autolysis at maturity (Fig. 1). Dalessandro and Roberts³ observed WVM formation when caffeine was used to inhibit cytokinesis. They considered their results to be inconclusive, however. Other references^{4,5} have been made to WVM formation without cell division but these reports have been based only on the relative sizes of WVM and surrounding undivided parenchyma cells.

I report here the differentiation of WVM without DNA synthesis, mitosis or cell division in cultured explants of Romaine lettuce (*Lactuca sativa* L. cv Romana). Cylindrical disks of parenchyma tissue, 5 mm in diameter and 1.5 mm thick, were isolated aseptically from the pith of Romaine lettuce heads and placed basal end down on a chemically defined medium⁶ containing the DNA synthesis inhibitor fluorodeoxyuridine (FUDR) (10^{-5} M). The numbers of WVM and parenchyma cells were assayed after 14 d of incubation.

Fig. 1 Immature WVM and parenchyma cell in Feulgen-stained squash preparation. Photographed with partially polarised illumination to highlight the birefringent secondary wall of the WVM. Both cells are nucleate (arrows). Bar represents 50 μm .



The results of a representative experiment are presented in Table 1. Disks placed on a medium without FUDR underwent extensive proliferation, as expected, producing approximately 25 times the original number of cells, 7% of the final cell number being WVM. In disks grown on medium with FUDR 6% of the cells differentiated into WVM without any apparent increase in cell number.

There was considerable variation in the number of WVM produced in the presence of FUDR; the average number per disk ranging between 0 and 9×10^3 . This variation was probably due to seasonal effects and to the commercial origin of the plant material. Results were, however, consistent within single experiments.

Table 1 Parenchyma cells and wound vessel members in tissue disks incubated with or without FUDR

	Parenchyma cells ($\times 10^3$)	Wound vessel members ($\times 10^3$)
0 d	87.9 $\pm$ 5.6	0
14 d (no FUDR)	2,240 $\pm$ 220	180 $\pm$ 13
14 d (10^{-5} M FUDR)	79.0 $\pm$ 3.8	4.7 $\pm$ 1.2

Parenchyma cells and WVM in tissue disks incubated with or without FUDR. Heads of Romaine lettuce were purchased at a local retail outlet, defoliated and surface-sterilised with 0.5% sodium hypochlorite. Cylinders of tissue 5.0 mm in diameter were removed from the centre of the core with a sterile cork borer and sliced into disks 1.5 mm thick. Disks were placed with their morphological basal end down on sterile medium⁶ containing indole-3-acetic acid (10 mg l^{-1}), kinetin (6-furfurylaminopurine, 0.1 mg l^{-1}) and 0.9% repurified Difco agar in 6-cm diameter Petri plates. FUDR was filter-sterilised and added to the medium when it had partially cooled. The tissue disks were cultured in the dark at $25 \pm 1^\circ \text{C}$ and at the end of the incubation period dispersed in chromic acid-HCl solution by drawing through a 22-gauge needle⁶. Cell counts were made in six 5- μl samples of an appropriate dilution of the macerate placed on a microscope slide.

Parenchyma cells of the pith are, *in situ*, quiescent and DNA synthesis does not begin for at least 20 h after isolation of the explants (unpublished). Although this seemed more than enough time for the uptake of FUDR in quantities sufficient to inhibit DNA synthesis, the effectiveness of the inhibitor was tested directly by microspectrophotometric measurement of the DNA content of Feulgen-stained nuclei (Fig. 2).

All nuclei of freshly isolated pith contained the 2C level of DNA, confirming earlier reports^{7,8} that cells of this species arrest exclusively in the G_1 phase of the nuclear cycle. In the nuclei of cells grown for 14 d on medium without FUDR the DNA content ranged between 2C and 4C. In FUDR-treated disks, however, there was no measurable DNA synthesis in the nuclei of parenchyma cells. The DNA content of nuclei in 50 immature WVM which had not yet undergone autolysis was also measured. These cells were unambiguously identified as WVM by the presence of secondary walls in the characteristic reticulate pattern (Fig. 1). The amount of nuclear DNA in WVM which had just begun to differentiate corresponded to the 2C level. As these cells continued to differentiate chromatin condensed, leading to an increase in self-absorption and slightly lower values of relative DNA content.

Mitosis and cell division could not have occurred in FUDR-treated disks since all cells were originally in G_1 and did not synthesise measurable amounts of DNA. To verify this conclusion, freshly isolated disks were placed on medium containing FUDR (10^{-5} M) and removed at 12 h intervals for the next 14 d. The disks were stained with Feulgen and squash preparations were systematically and thoroughly examined. WVM were first detected after 5 d of incubation and reached a maximum of approximately 2×10^3 per disk by day 12. No mitotic figures were found in any of the preparations.

The Romaine lettuce-xylogensis system is particularly significant in that the cells which differentiate are apparently uncommitted to the formation of vessel members before isolation. The pith tissue of this species is composed of a

homogeneous population of parenchyma cells devoid of vascular tissue. Xylem elements form only as a response to injury and do not normally differentiate in the pith during the life cycle of an undisturbed plant. In seedlings of wheat germinated from gamma-irradiated grain, differentiation of complex tissues, including xylem, takes place without DNA synthesis⁹. But vessels arise only in procambial tissue already formed in the embryo and presumably committed to differentiation¹⁰. Similarly, there have been reports that, in appropriate conditions, determined mammalian¹¹ and insect^{12,13} cells may differentiate without DNA replication.

Evidence linking differentiation to a previous round of cell division has often led to the suggestion that events during the cell cycle, especially in the DNA replication phase, are themselves integral components of a genetic reprogramming mechanism (reviewed in ref. 14). While most plant and animal cell differentiation undoubtedly occurs after cell division, the experimental uncoupling of these processes suggests that this is not a causal association. In the meristem, cell doubling may be an added constraint on differentiation to ensure that progenitor cells are reproduced before differentiation occurs. A homeostatic mechanism of this nature would protect the integrity of the progenitor cell line *in vivo* and may be responsible for the frequently observed coincidence of cell division and

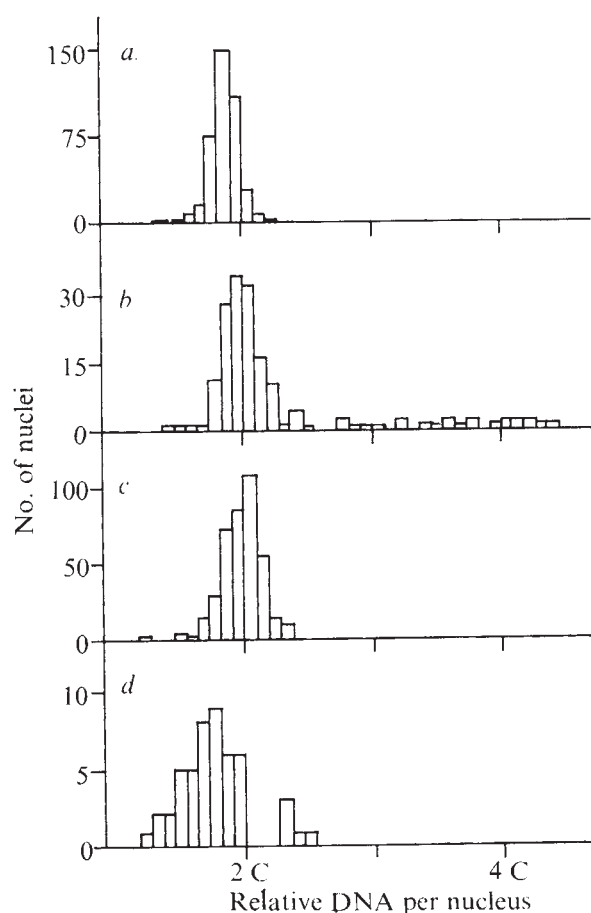


Fig. 2 Relative amounts of DNA per nucleus before and after 14 d of incubation (Table 1). *a*, Freshly isolated parenchyma cells, sample size (N) 400. *b*, Parenchyma cells after 14 d incubation no FUDR in medium, $N = 165$. *c*, Parenchyma cells after 14 d incubation, 10^{-5} M FUDR, $N = 400$. *d*, Immature WVM after 14 d incubation, 10^{-5} M FUDR, $N = 50$. Disks of tissue were fixed in ethanol-acetic acid (3:1 v/v), hydrolysed in 5N HCl for 60 min at room temperature and stained by the Feulgen method. The stained preparations were placed in a 5% pectinase solution for 2 h and drawn through a 22-gauge needle to separate cells before mounting in Canada balsam. Measurements of relative DNA per nucleus were made with a Zeiss scanning microspectrophotometer at 560 nm and normalised with readings of half telophase and prophase mitotic figures taken to be 2C and 4C values, respectively.

differentiation *in vitro*. Such a mechanism might also operate where animal cells differentiate in proliferating tissue.

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ROBERT TURGEON

Rockefeller University,
New York, New York 10021

Received August 1; accepted August 26, 1975.

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Stimulation of phosphorylation and inactivation of pyruvate dehydrogenase by physiological inhibitors of the pyruvate dehydrogenase reaction

OXIDATION of pyruvate in perfused rat heart is inhibited by diabetes and by oxidation of fatty acids and ketone bodies¹. Enhanced release and oxidation of fatty acids from muscle glycerides may be an important factor in the effects of diabetes²⁻⁴. These effects on pyruvate oxidation have generally been attributed to inhibition of pyruvate dehydrogenase, and regulation of this enzyme is considered to be of crucial importance in the selection of fuels for respiration and in the metabolic changes in heart and other tissues in diabetes and starvation.

Mammalian pyruvate dehydrogenases contain three enzymes, pyruvate decarboxylase (E_1), dihydrolipoate acetyl

transferase (E_2) and dihydrolipoate dehydrogenase (E_3) bound together in a single complex. They catalyse the conversion of pyruvate, CoA and NAD into acetyl CoA, NADH₂ and CO₂ in the presence of Mg²⁺ and thiamine pyrophosphate (TPP) by a coordinated series of reactions in which lipoate bound covalently to the acetyl transferase visits active sites on the three enzymes sequentially. Lipoate reacts with hydroxyethyl TPP formed on E_1 to give acetyl hydrolipoate which reacts with CoA on E_2 to form acetyl CoA and dihydrolipoate; the latter then reacts with NAD on E_3 to form NADH₂ and regenerate lipoate⁵. Reversible reactions are catalysed by E_2 and E_3 whereas the reaction catalysed by E_1 is essentially irreversible. NADH₂ can thus form dihydrolipoate; and acetyl CoA plus NADH₂ can form acetyl hydrolipoate and hydroxyethyl TPP within the complex by reversal of reactions catalysed by E_2 and E_3 .

Pyruvate dehydrogenase in heart muscle or liver is subject to end product inhibition by acetyl CoA (competitive with CoA) and NADH₂ (competitive with NAD) and this inhibition is presumed to be due to accumulation of acetyl hydrolipoate and dihydrolipoate within the complex^{6,7}. Mammalian pyruvate dehydrogenases are also regulated by pyruvate dehydrogenase kinase within the complex, which catalyses phosphorylation and inactivation of pyruvate decarboxylase with ATP-Mg²⁺; a phosphatase catalyses dephosphorylation and reactivation^{8,9}. In perfused heart oxidation of fatty acids or ketone bodies leads to a substantial increase in the concentration ratio of acetyl CoA-CoA. With acetate, for example, this ratio increases sixtyfold and oxidation of pyruvate is almost totally suppressed within 1 min of entry of acetate into the coronary circulation^{10,11}. It has been suggested that this change in concentration ratio may lead to end product inhibition of pyruvate dehydrogenase and thus contribute to inhibition of pyruvate oxidation. In perfused heart oxidation of fatty acids and ketone bodies also stimulates phosphorylation and inactivation of pyruvate dehydrogenase^{12,13}. The mechanism of this effect is not known but it presumably involves activation of the kinase reaction or inhibition of the phosphatase reaction or both.

The known effectors of the kinase reaction (ADP, pyruvate, pyrophosphate compounds, Ca²⁺ and Mg²⁺ as inhibitors) and of the phosphatase reaction (Mg²⁺ and Ca²⁺ as activators^{8,9,14,15}) did not provide an obvious explanation of effects of fatty acids and ketone bodies in the perfused

Table 1 Effects of NAD, NADH₂, CoA and acetyl CoA on activity of pig heart pyruvate dehydrogenase kinase

Concentration (mM)				Pyruvate dehydrogenase kinase activity		
NAD	NADH ₂	CoA	acetyl CoA	(mean ± s.e., counts per 10 min in protein-bound phosphate)		
0	0	0	0	3,812 ± 47 (12)	Effect of NAD, NADH ₂ and ratio of NADH ₂ -NAD	
2	0	0	0	3,753 ± 42 (3)		
0	0.01	0	0	4,853 ± 104 (3)		
2	0.01	0	0	3,958 ± 26 (3)		
0	0.5	0	0	4,830 ± 91 (3)		
2	0.5	0	0	4,113 ± 96 (3)		
2	0.01	0	0	3,958 ± 26 (3)	Effect of CoA	
2	0.01	0.25	0	2,922 ± 51 (3)		
2	0.01	2.0	0	2,148 ± 35 (3)		
2	0.5	0	0	4,113 ± 96 (3)		
2	0.5	0.25	0	3,518 ± 66 (3)		
2	0.5	2.0	0	2,593 ± 26 (3)		
2	0.01	0	0	3,958 ± 26 (3)	Effect of acetyl CoA	
2	0.01	0	0.25	4,561 ± 41 (3)		
2	0.5	0	0	4,113 ± 96 (3)		
2	0.5	0	0.25	4,613 ± 17 (3)		
2	0.5	2.0	0	2,593 ± 26 (3)		
2	0.5	2.0	0.25	3,884 ± 82 (3)		
2	0.5	2.0	0.5	4,282 ± 98 (3)	Effect of ratio of acetyl CoA-CoA	
2	0.5	2.0	1.0	4,607 ± 54 (3)		

Pig heart pyruvate dehydrogenase (2 U ml⁻¹) was incubated at 30 °C for 1.5 min in 20 mM potassium phosphate, 5 mM 2-mercaptoethanol, pH 7.0 (volume 50 µl) with γ-³²P-ATP (100 µM, 8 µCi µmol⁻¹) and 2 mM MgCl₂ with other additions as shown. The reaction was terminated and protein-bound phosphate assayed in 30 µl samples as in ref. 15. The number of assays is shown in parentheses.

heart. It seemed likely that the concentration ratios of acetyl CoA-CoA and of NADH₂-NAD might modulate kinase or phosphatase reactions in addition to their effects on the dehydrogenase reaction. No consistent and significant effects of NAD, NADH₂, acetyl CoA or of CoA on the phosphatase reaction¹⁶ have been detected. Attempts to demonstrate effects of the concentration ratio of acetyl CoA-CoA on the kinase reaction yielded very variable results¹⁵. Recent studies of elementary reactions in the pyruvate dehydrogenase complex suggested that the concentration ratio of NADH₂-NAD might be critical to a demonstration of effects of the concentration ratio of acetyl CoA-CoA on the kinase reaction.

This expectation has been realised in the present study in which pyruvate dehydrogenase kinase activity has been measured by the incorporation of ³²P into protein-bound phosphate from γ -³²P-ATP by an assay procedure described previously¹⁵. The only variation in the present study has been addition of effectors immediately before initiation of the reaction with γ -³²P-ATP. As shown by representative data in Table 1, NADH₂ activates the kinase reaction and NAD reverses the NADH₂ activation. In the presence of mixtures of NADH₂ and NAD, which themselves only partially activate, acetyl CoA activates the kinase reaction and CoA inhibits. Acetyl CoA reverses the inhibition by CoA provided sufficient NADH₂ is present. The pyruvate dehydrogenase kinase reaction is thus sensitive to concentration ratios of NADH₂-NAD and of acetyl CoA-CoA in a manner which may provide a basis for effects of fatty acids and ketone bodies also stimulates phosphorylation and inactivation of pyruvate dehydrogenase in perfused heart.

The mechanism of these complex effects of NADH₂, NAD, CoA and acetyl CoA is not known. In pyruvate dehydrogenase, E₂ forms the core of the molecule and carries E₁, E₃ and pyruvate dehydrogenase kinase¹⁷. If NADH₂, NAD, acetyl CoA and CoA affect the kinase reaction through binding to their substrate sites on E₂ and E₃, their effects must be transmitted from these subunits to the kinase or its substrate (E₁). One possibility is that lipoate might transmit these effects because it visits active sites on E₁, E₂ and E₃; the presence of oxidised lipoate on E₁ might inhibit phosphorylation of E₁ by the kinase so that when lipoate in one of its forms is located on E₂ or E₃ the kinase reaction could be facilitated. More conventional explanations for regulatory interactions between subunits involving either binding of the effectors to their substrate sites on E₂ and E₃ or to additional regulator sites on E₁ or the kinase may provide an alternative explanation for these effects of NADH₂, NAD, acetyl CoA and CoA.

Since this manuscript was submitted, Pettit *et al.*¹⁸ have reported inhibition of beef heart pyruvate dehydrogenase kinase reaction by CoA and by NAD, and activation of the kinase reaction by NADH₂ and by acetyl CoA. Our results with pig heart complex provide confirmation for inhibition by CoA and activation by NADH₂. We have not detected inhibition by NAD but rather reversal of NADH₂ activation by NAD. As mentioned earlier, we have not been able to detect significant activation of the kinase reaction by acetyl CoA except in the presence of mixtures of NAD plus NADH₂ which themselves produce no or only partial activation of the kinase reaction.

RONALD H. COOPER
PHILIP J. RANDLE
RICHARD M. DENTON

M101, Department of Biochemistry,
Medical School, University of Bristol,
University Walk, Bristol BS8 1TD, UK

Received July 25; accepted September 18, 1975.

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Pregnancy in the hyrax and erythrocyte metabolism of progesterone

HYRAXES are unusual for small mammals because they have a long gestation period (7.5 months) for their size (2-3 kg) and intra-abdominal testes^{1,2}. Hystricomorph rodents with long gestation periods relative to mature weight have a pregnancy-specific plasma protein with a high affinity for progesterone—progesterone-binding globulin (PBG). It occurs at high concentrations in the guinea pig and coypu, and evidently reduces the rate of progesterone clearance from blood³. We have found that, in the hyrax, the progesterone concentration in the corpus luteum is appreciable whereas that in plasma is low, and that erythrocytes metabolise progesterone *in vitro*. The results of this study are relevant to the phylogenetic affinity of the hyrax and elephant, and there is doubt about whether progesterone is the hormone of pregnancy in the elephant, since its concentration in the corpus luteum and peripheral plasma has been found to be very low⁴⁻⁷.

Steroid hormones of pregnancy were measured in the rock hyraxes, *Heterohyrax brucei* and *Procavia habessinica*, shot near Nairobi, and in captive *H. brucei*. Blood of shot specimens was taken into heparinised syringes and the ovaries and placentae removed immediately; all samples were transported to the

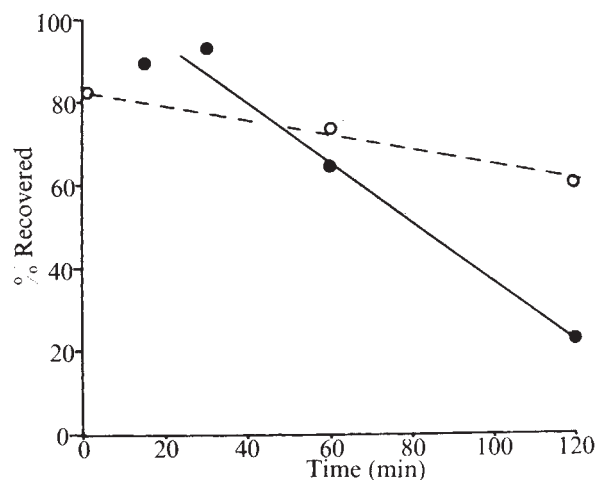


Fig. 1 The metabolism and uptake of ³H-progesterone by hyrax blood. Whole blood (0.5 ml) was incubated with ³H-progesterone, 0.5 μ Ci, 2.8 ng, at 4 °C (○) and 37 °C (●). Labelled progesterone was extracted from blood with 5 ml diethyl ether after the addition of 100 μ g unlabelled steroid to correct for procedural losses. After extraction, progesterone was separated by thin-layer chromatography (75:25, benzene-ethyl acetate), eluted with ethanol, and its specific activity determined by ultraviolet spectrophotometry and liquid scintillation spectrometry. The amount of labelled progesterone recovered was corrected for procedural losses and the result expressed as a percentage of added ³H-progesterone. The metabolism of ³H-progesterone at 4 °C was negligible and the apparent loss of steroid was related to red cell uptake. Enzymatic metabolism at 37 °C, however, was substantial.

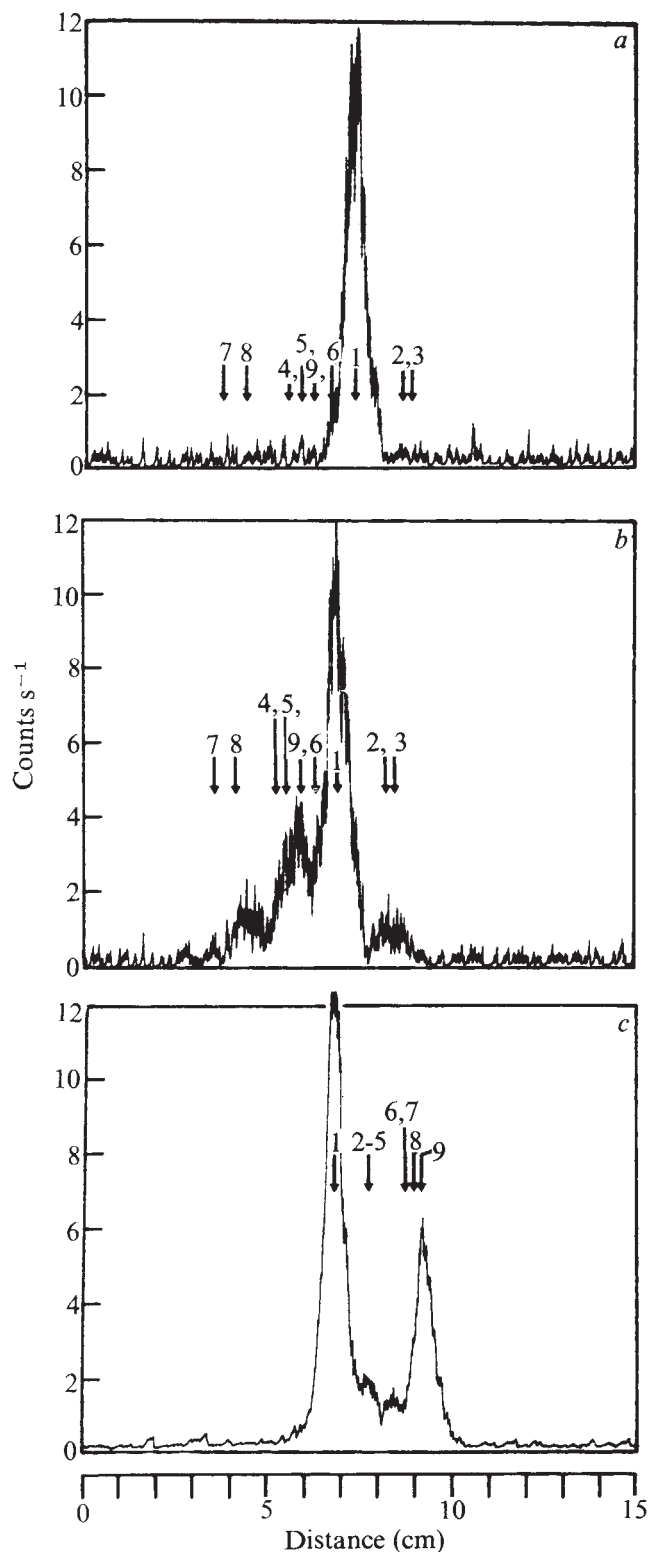


Fig. 2 The metabolism of ^3H -progesterone by hyrax blood. Saline or whole blood (5 ml) was incubated with 2.5 μCi ^3H -progesterone (14 ng) at 37°C for 3 h. After incubation, 1 ml 2.5% NaOH was added and each sample was extracted with 25 ml diethyl ether which was washed with distilled water and evaporated to dryness. The samples were applied to thin-layer chromatography plates and run for 15 cm in the solvent system, benzene-ethyl acetate, 4:6. *a*, ^3H -Progesterone added to saline; *b*, ^3H -progesterone added to hyrax blood; *c*, ^3H -compounds recovered from plate *b* acetylated at 70°C for 30 min. Chromatographic mobility of marker steroids indicated: (1) progesterone, (2) 5α -pregnane-3,20-dione; (3) 5β -pregnane-3,20-dione; (4) 20α -hydroxypregn-3-one; (5) 20β -hydroxypregn-3-one; (6) 3β -hydroxypregn-20-one; (7) 5β -pregnane-3 α ,20 α -diol (pregnanediol); (8) 5β -pregnane-3 α ,20 β -diol; (9) 5β -pregnane-3 β ,20 β -diol. Note the metabolism of ^3H -progesterone by hyrax blood (*b*) and the formation of compounds with chromatographic mobilities similar to those of 5α - and 5β -pregnanediol, and of hydroxylated pregnane metabolites with chromatographic properties similar to those of dihydroxypregnane compounds after acetylation.

progesterone (Table 1). In *Heterohyrax* the luteal concentration in pregnancy was $5.8\text{--}24.2\text{ ng mg}^{-1}$, and one pregnant *Procavia* had 18.6 ng mg^{-1} . Progesterone concentration was low in one animal (J5, Table 1) but this did not seem to be associated with a failing pregnancy. A very low progesterone concentration was found in residual ovarian tissue after the removal of luteal tissue (less than 0.1 ng mg^{-1}) and in the placenta (less than 0.002 ng mg^{-1}).

An unusual feature of the hormone content of the hyrax corpus luteum was the high concentration of immunoreactive unconjugated oestrogens, $35\text{--}1,840\text{ pg mg}^{-1}$ (Table 1). In contrast, the oestrogen concentration in residual ovarian tissue did not exceed 8 pg mg^{-1} , and in placental tissue it was less than 0.4 pg mg^{-1} .

Plasma concentrations of progesterone in blood taken by cardiac puncture were low in all females ($1.2 \pm 0.3\text{ ng ml}^{-1}$, $n = 22$). In immature females the concentration was 0.3 and 0.4 ng ml^{-1} , in non-pregnant females, $0.6 \pm 0.2\text{ ng ml}^{-1}$, and in pregnant females values ranged from less than 0.1 to 4.6 ng ml^{-1} during early, mid and late gestation. In hyrax J16 (Table 1), a mature female housed in laboratory conditions, the concentration in weekly samples varied between 1.5 and 3.8 ng ml^{-1} during approximately 30 d before a fertile mating. In early pregnancy, 4.6 ng ml^{-1} was recorded. The plasma progesterone concentration in males was $0.6 \pm 0.3\text{ ng ml}^{-1}$ ($n = 8$).

The plasma concentration of unconjugated oestrogens in immature females was less than 10 pg ml^{-1} , in non-pregnant females 138 ± 19 ($n = 19$), and in pregnant females $106 \pm 44\text{ pg ml}^{-1}$ ($n = 5$). The concentration in males, however, was of the same order, $134 \pm 35\text{ pg ml}^{-1}$ ($n = 8$), indicating that reactivity was probably nonspecific.

Plasma samples from shot specimens contained a lower concentration of progesterone than those from captive animals obtained under anaesthesia. This loss of activity after sampling seemed to be related to a delay in separating the plasma from shot specimens. ^3H -progesterone incubated with whole blood at 37°C was metabolised (Fig. 1) to compounds corresponding in mobility to ring A saturated compounds less polar than progesterone, and hydroxylated steroids more polar than progesterone (Fig. 2). After incubation at 4°C only 62% of the ^3H -progesterone was recovered and the loss was associated with uptake by the red cells rather than enzymatic metabolism. Blood from shot specimens taken within 5 min of death and extracted immediately with diethyl ether gave progesterone values of 1.1 and 1.8 ng ml^{-1} (corrected for procedural losses) in two early-mid-pregnant females. These values were similar to those obtained in plasma from captive animals, and indicated that the amount of progesterone sequestered in erythrocytes was not large.

The amount of ^3H -progesterone recovered from hyrax blood after a 120-min incubation at 37°C was similar in non-pregnant and pregnant hyraxes (range 0.8–13.3%). The metabolism and uptake was attributable to erythrocytes since incubations with

laboratory at 4°C . Blood was taken from captive animals by cardiac puncture under chloroform anaesthesia. Plasma was separated by centrifugation within 15 min of collection from these animals, but at least 3 h elapsed before plasma could be obtained from shot specimens. Plasma and tissue samples were either extracted immediately or stored at -15°C . Progesterone and total unconjugated oestrogens were determined by radioimmunoassay (method A in ref. 8 and ref. 9). Tissue progesterone concentrations were corrected for procedural losses assessed by the recovery of a tracer amount of ^3H -progesterone added to each tissue. Details of these procedures will be published later.

The corpus luteum during pregnancy varied in wet weight between 13 and 18 mg and contained a significant amount of

Table 1 Concentration of progesterone and total unconjugated oestrogens in the corpus luteum of *Heterohyrax brucei*

Animal no.	Condition of animal	No. of foetuses and distribution	Weight of foetus (g); C-R length (mm)	Mean wet weight (mg)*	Progesterone concentration (ng mg ⁻¹)	Corpus luteum Progesterone content (ng)	Oestrogen concentration (pg mg ⁻¹)	Oestrogen content (ng)
J12	Non-pregnant	—	—	16 (2)	26.1	417.6	682	10.9
J16	Early pregnancy	2 (1L, 1R)	2.65†	18 (3)	14.8	266.4	35	0.6
J13‡	Early pregnancy	2 (1L, 1R)	—	13.5 (2)	18.6	251.1	139	1.9
D6	Early-mid-pregnancy	2 (1L, 1R)	10; 75	16 (2)	23.3	372.8	650	10.4
D4	Early-mid-pregnancy	2 (1L, 1R)	10; 75	14 (2)	24.2	338.8	1,840	25.8
J2	Mid-pregnancy	2 (1L, 1R)	25; 98	14 (2)	15.9	222.6	820	11.5
J5	Mid-pregnancy	2 (1L, 1R)	35; 110	13 (2)	5.8	156.6	620	16.7
J4	Late pregnancy	1 (1L)	70; 140	14 (2)	21.6	302.4	1,600	22.4

*Numbers in parentheses indicate number of corpora lutea.

†Weight of conceptus

‡*Procavia* sp.

an equivalent volume of red cells resulted in recoveries of only 11.0 and 12.0% of added ³H-progesterone. The rate of disappearance of ³H-progesterone was reduced if the erythrocytes had been lysed with distilled water; the average amount of labelled progesterone recovered being 41.1%. The enzymatic activity of erythrocytes with respect to ³H-progesterone was abolished by the addition of NaF.

The progesterone requirements of pregnancy may be met in various ways in different mammals¹⁰. In rats, sheep and women there is an increased production of progesterone; in guinea pigs there is a marked decrease in its rate of clearance and only a modest rise in production. The hyrax does not seem to fit either of these categories, since no evidence has been found of a high concentration of plasma progesterone in gestation, or of a pregnancy-specific plasma protein with a high affinity for progesterone.

The ability of erythrocytes to metabolise steroid hormones, including progesterone, has been reported in several species including man¹¹⁻¹⁵, but only in the foetal lamb, calf and kid has a rapid conversion of progesterone to 20 α -dihydroprogesterone been found^{14,15}. Although the two studies were not directly comparable, the rate of erythrocyte metabolism of progesterone *in vitro* seemed to be less in the hyrax than in the foetal lamb. In both species, however, cell lysis reduced enzymatic activity. In the hyrax, NaF, an inhibitor of anaerobic glycolysis, blocked progesterone metabolism which further supports the suggestion that steroid dehydrogenases in red cells utilise NADH generated by the glycolytic pathway¹².

A low plasma progesterone concentration during pregnancy has also been reported in the tammar wallaby¹⁶, but these marsupials have a gestation period of only 28 d. Maintenance of pregnancy by low plasma progesterone levels in the hyrax may be facilitated by a particularly sensitive receptor mechanism in the target tissues, with blood metabolism contributing to the protection of maternal and foetal tissues from any adverse effects of high hormone concentrations. There remains the controversial but interesting possibility that support of gestation in these animals depends on a metabolite, perhaps associated with erythrocyte metabolism of progesterone, rather than on progesterone itself. This possibility recalls work on the elephant which has cast doubt on the role of progesterone as the hormone of pregnancy⁷. Although the progesterone concentration in the corpus luteum of the hyrax contrasts with the low level reported in the elephant, the plasma levels in both animals are low during gestation. In neither animal does the endocrinology of pregnancy conform to patterns described in other mammals.

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R. B. HEAP
S. GOMBE

Department of Animal Physiology,
University of Nairobi, and
ARC Institute of Animal Physiology,
Babraham, Cambridge CB2 4AT, UK

J. B. SALE

Department of Zoology,
University of Nairobi,
PO Box 30197, Nairobi, Kenya

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Pressure reversal of narcosis produced by anaesthetics, narcotics and tranquillisers

The first demonstration of pressure reversal of anaesthesia in amphibians was provided by the tadpole experiments of Johnson and Flagler¹ with ethanol and ethyl carbamate. Since then the phenomenon has been studied using general anaesthetics in newts, mice and marine organisms^{2,3}, but few of these agents have been studied in detail and the results in some cases, for example, for barbiturates^{4,5}, are conflicting. Another aspect of the interaction of pressure and anaesthesia is the amelioration of the high pressure nervous syndrome by nitrous oxide, hydrogen or nitrogen⁶⁻⁸. It is not, however, known if other agents can also give 'pressure protection', and tadpoles provide a relatively easy model for a preliminary screening of potentially suitable drugs. We have studied both these aspects of the interaction between pressure and narcosis using intravenous, local and inhalational general anaesthetics and tranquillisers. We show that pressure reversal occurred with all our agents pro-

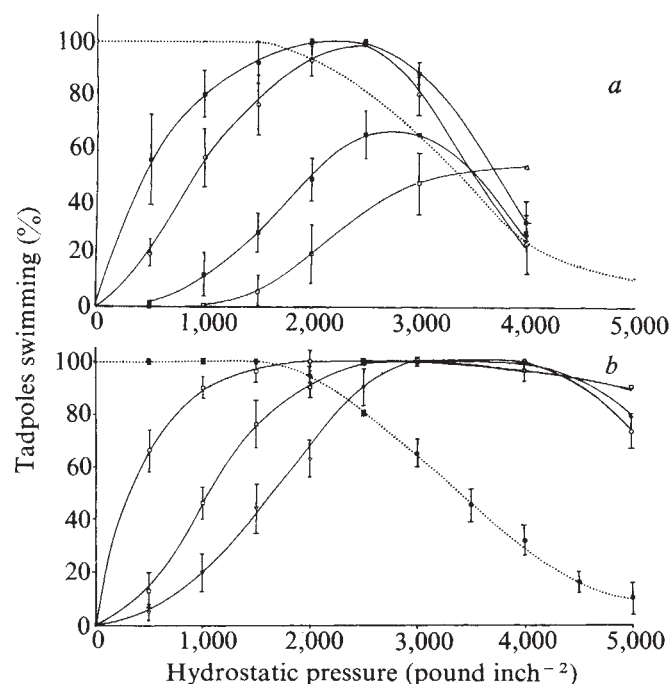


Fig. 1 *a*, Effect of pressure on tadpoles swimming in water equilibrated with different concentrations of halothane. ●, 0.005 atm; ○, 0.0075 atm; ■, 0.01 atm; □, 0.015 atm. Error bars indicate ± 1 s.e.m. calculated from five groups of five tadpoles. Dotted line shows effect of pressure alone. The tadpoles (*Rana temporaria*) were immersed in an aqueous solution of the agent at a known partial pressure and equilibrated for up to 30 min before pressurising to avoid any transitory effects associated with uptake and distribution of the agent. The chamber (400 ml capacity) was completely filled with solution which provided sufficient oxygen throughout the experimental period (up to 15 min). Pressure was applied using an oil-filled pump connected by way of a side chamber to prevent contamination. In almost all cases the observed effects were completely reversible, although recovery could take up to 18 h. *b*, Effect of pressure on tadpoles equilibrated with althesin—an intravenous steroid anaesthetic consisting of alphaxalone (3 parts by weight), alphadalone acetate (1 part by weight) in cremophor E. L. plus saline; (Cremophor E. L. was tested alone to ensure its inactivity). Concentrations were expressed as total steroid in $\mu\text{g ml}^{-1}$. ●, No anaesthetic present (dotted line); ○, 3.0 $\mu\text{g ml}^{-1}$; □, 4.2 $\mu\text{g ml}^{-1}$; ▽, 6.0 $\mu\text{g ml}^{-1}$. Error bars indicate ± 1 s.e.m. calculated from five groups of five tadpoles.

ducing narcosis in tadpoles, whereas pressure protection does not seem to be as universal.

Tadpole swimming activities in the presence of increased hydrostatic pressure with or without halothane or althesin are shown in Fig. 1*a* and *b*. It was only possible to quantify the response in terms of the presence or absence of movement (which was either spontaneous or initiated by tapping the side of the chamber). With pressure alone there was a progressive (but reversible) decrease in swimming activity—termed ‘pressure paralysis’—when the tadpoles became generally flaccid. In contrast to the inactivity produced by narcosis, however, pressure paralysis was normally accompanied by a pronounced bending of the tail. Other pressure effects included hyperactivity at 1,000–1,500 pound inch⁻² and tremors at 3,000–4,000 pound inch⁻².

The tadpoles were initially inactive in the presence of narcotics, but as the hydrostatic pressure increased they resumed swimming. The variation of narcotic reversal pressure with narcotic dose gives some indication of the differing degrees of pressure reversal. We found that the magnitude of the effect was too large to be attributed solely to the expected change of solubilities with pressure⁹. Decompression at the point of narcotic reversal produced an immediate return to narcosis which was again reversed by recompression. For both agents the effect of increasing the pressure was biphasic, although with althesin pressure paralysis only occurred above 5,000 pound

inch⁻². At the two highest halothane partial pressures the animals never fully recovered from narcosis before the onset of pressure paralysis.

Other agents were investigated using groups of five tadpoles and the results, expressed in terms of the onset of narcotic reversal and pressure paralysis at different doses of agents, are shown in Tables 1 and 2. The agents listed in Table 2 are not normally considered to be satisfactory general anaesthetics but, when the doses are sufficient to cause narcosis in tadpoles, pressure reversal can still occur. It has been postulated that benzocaine and procaine produce their local anaesthetic effects by membrane expansion, whereas agents such as lignocaine act at specific charged sites¹⁰. All three local anaesthetics however, had their narcotic effects reversed by pressure, which is consistent with the hypothesis that there is expansion of the critical site for narcosis independent of the agent.

Table 1 Narcotic reversal and pressure paralysis in tadpoles influenced by clinical anaesthetics

Agent	'Dose'	Complete narcotic reversal (atm)	Complete pressure paralysis (atm)
Methoxyflurane	0.001 atm	51	272
	0.002 atm	204	340
Chloroform	0.0025 atm	34	306
	0.005 atm	170*	272
Halothane	0.005 atm	102	306
	0.0075 atm	170	306
	0.01 atm	187*	306
	0.015 atm	238*	340†
Trichloroethylene	0.005 atm	68	306
	0.0077 atm	102*	272
Enflurane	0.008 atm	102	374
	0.015 atm	170	374
Diethyl ether	0.01 atm	68	408
	0.02 atm	136	> 408
Cyclopropane	0.1 atm	102	306
	0.15 atm	136	306
Nitrous oxide	0.8 atm	51	408
Althesin	3 $\mu\text{g ml}^{-1}$	136	> 408
	4.2 $\mu\text{g ml}^{-1}$	170	> 408
	6 $\mu\text{g ml}^{-1}$	204	> 408
Methohexitone	0.1 mM	102	272
	0.13 mM	102	306
	0.17 mM	238	340
‡ Propanidid	0.89 mM	102	> 272
‡ Thiopentone	1.9 mM	136	340

* Pressure at which maximum response was obtained when complete pressure reversal did not occur.

† These animals did not recover at the end of the experiment.

‡ Lethal – narcotising dose ratio close to unity.

Our results with ethanol are in agreement with those of Johnson and Flagler¹, who used different species of tadpoles and larvae. A high dose of morphine produced tadpole narcosis but there was no effect with two other narcotic analgesics, fentanyl citrate and pentazocine. The pressure reversal of the narcosis produced by the different tranquillisers emphasises that this phenomenon is not associated with specific agents or groups of drugs.

When studying the amelioration of the high pressure nervous syndrome we noted that tremors never occurred in the presence of narcotics, but since this effect was difficult to quantitate, we have not included it in our characterisation of ‘pressure protection’. With pressure alone, 100% activity is lost above 1,470 pound inch⁻², which is equivalent to 100 atm. Halothane seemed to give no significant protection against the paralyzing effects of high pressure (Fig. 1*a*); the animals exposed to 0.015 atm halothane died at 330 atm. In Tables 1 and 2 any figure for complete narcotic reversal above 100 atm indicates some degree of pressure protection. Several agents protected the tadpoles to some extent against the effects of very high pressure; these have a pressure paralysis value of > 300 atm. Althesin was the most successful agent that we used and is shown in Fig. 1*b*. This protective effect of althesin does not

Table 2 Narcotic reversal and pressure paralysis in tadpoles influenced by unconventional anaesthetics

Agent	'Dose'	Complete narcotic reversal (atm)	Complete pressure paralysis (atm)
†Lignocaine (pH 6.95)	1 mM	68	272
†Procaine (pH 7.20)	0.88 mM	34	238
Benzocaine (pH 7.23)	0.24 mM	34	306
Ethanol	0.43 M	68	272
	1.37 M	136	272
†Morphine	0.55 mM	34	204
†Chlorpromazine	0.16 mM	102	238*
Diazepam	0.09 mM	102	272
	0.18 mM	238	272*
†Droperidol	0.33 mM	68	272
†Promazine	0.31 mM	68	272*

*These animals did not recover at the end of the experiment.

†Lethal - narcotising dose ratio close to unity.

seem to apply to all steroids; for example, hydrocortisone, a non-anaesthetic steroid, gave no pressure protection.

Our results demonstrate that the phenomenon of pressure reversal applies to narcosis in tadpoles regardless of the agent used. The compounds included a wide range of drugs with a very large variation in both anaesthetic potency and molecular size. These results may be relevant to the analogous situation in mammals since the ratio of tadpole narcotic concentration to the AD_{95} for surgical anaesthesia in man¹¹ is 2.75 ± 0.62 (s.d.), which has a coefficient of variation within that predicted by the accuracy of both measurements. Pressure reversal occurring with the steroids in althesin is particularly interesting because their anaesthetic activity in mammals seems to be related to their stereochemistry, and some workers have assumed that their mode of action is fundamentally different from the inhalational agents¹². Our data suggest that, although there may be some underlying minor differences in the physico-chemical basis of the expansion at the site of action, there are no intrinsic differences in the molecular mechanism of narcotic action of the inhalational and intravenous agents we studied.

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M. J. HALSEY
BRIDGET WARDLEY-SMITH

Division of Anaesthesia,
Clinical Research Centre,
Watford Road,
Harrow HA1 3UJ, UK

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A leukaemic cell mutant with a thermolabile alanyl-transfer RNA synthetase

It may be of great help in the elucidation of regulatory systems in mammalian cells if we could isolate temperature-conditional mutants and identify their mutated functions. I report here that

a temperature-sensitive mutant of L5178Y murine leukaemic cells has a thermolabile L-alanyl-tRNA synthetase.

The *ts3* cells have been shown to exhibit a mutant character when grown in Fischer's medium but not in Ham's F12 medium, that is, cells grow at the non-permissive temperature (39 °C). L-alanine (10^{-4} M), hypoxanthine (3×10^{-5} M), and sodium pyruvate (10^{-3} M) are components of Ham's F12 medium but not Fischer's, and are responsible for growth of the mutant cells at the elevated temperature¹. Each of these substances was examined at various concentrations for growth stimulation.

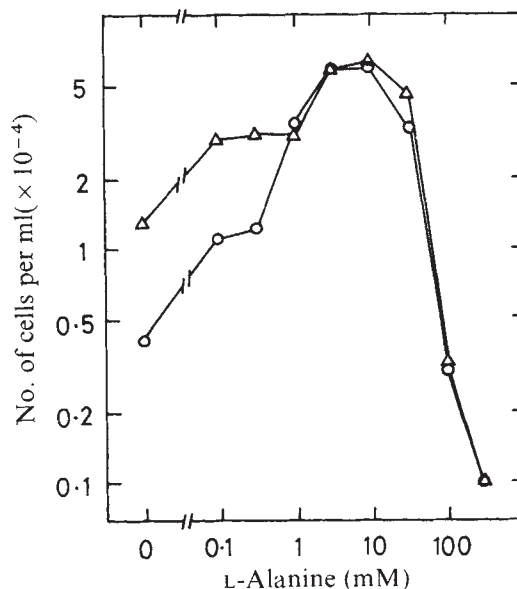


Fig. 1 Effect of L-alanine on growth of *ts3* cells at 39 °C. 1×10^4 *ts3* cells ml^{-1} were inoculated in dialysed calf serum-containing Fischer's medium supplemented with various concentrations of L-alanine with (Δ) or without ($\circ$) hypoxanthine (3×10^{-5} M) and sodium pyruvate (10^{-3} M). After incubation at 39 °C for 3 d the cell numbers were scored.

For hypoxanthine and pyruvate the optimal concentrations were the same as those contained in Ham's F12 medium. Varying concentrations of alanine, however, exerted great influence on cell growth. As shown in Fig. 1, at 0.1 and 0.3 mM L-alanine, both hypoxanthine and pyruvate are required for *ts3* cells to grow at 39 °C. Above concentrations of 1 mM L-alanine, hypoxanthine and pyruvate are dispensable and alanine alone can support growth. No other L-amino acids, nor D-alanine, nor β -alanine could be substituted for L-alanine. Cell growth was inhibited at concentrations higher than 30 mM alanine. In the lower range of alanine concentrations pyruvate may serve as a precursor of alanine and be converted to the amino acid in the cell. Hypoxanthine has been shown to have a growth-promoting effect for L5178Y cells (T. Shiomi, personal communication).

Since *ts3* cells were shown to require alanine for growth at 39 °C, I examined the possibility that alanine biosynthesis was impaired at a restrictive temperature in the mutant. The main pathway of alanine synthesis is a conversion of pyruvate and L-glutamate to L-alanine and α -ketoglutarate and the reaction is carried out by alanine aminotransferase (EC 2.6.1.2). The enzyme activity was determined after exposing cell extracts to a high temperature². The initial specific activities expressed as μmol pyruvate formed per min per mg protein were 14.0 and 15.2 and the residual activities after 60 min at 40 °C were 11.6 and 12.8 for wild-type and *ts3* cells, respectively. This shows that the enzyme activity of *ts3* cells was as stable as that of parental wild-type cells at the elevated temperature, the residual activities being more than 80% for both wild-type and mutant cells. This result indicates that the main biosynthetic pathway of alanine is not altered in *ts3* cells.

The rates of incorporation of radioactive amino acids and

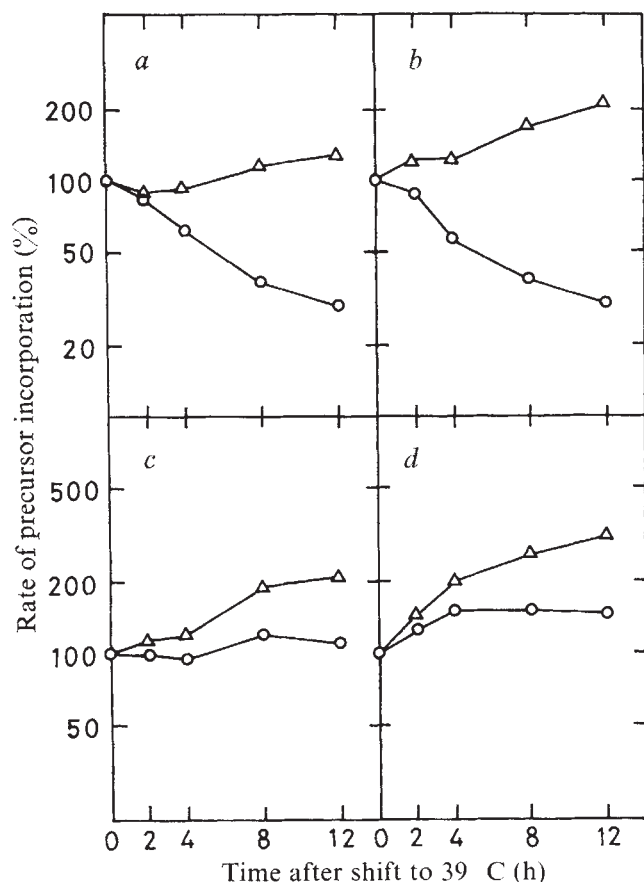


Fig. 2 Rates of incorporation of radioactive amino acids and nucleosides into acid-precipitable material after shift from 33 to 39 °C for *ts3* (○) and wild-type (Δ) cells. At the indicated times, cells were labelled for 30 min with either: *a*, ^{14}C -alanine (159 Ci mol^{-1} , $0.8 \mu\text{Ci ml}^{-1}$); *b*, ^{14}C -thymidine (49.5 Ci mol^{-1} , $0.1 \mu\text{Ci ml}^{-1}$); *c*, ^{14}C -valine (150 Ci mol^{-1} , $0.2 \mu\text{Ci ml}^{-1}$) or *d*, ^{14}C -uridine (59 Ci mol^{-1} , $0.01 \mu\text{Ci ml}^{-1}$). Ice-cold 5% TCA-insoluble material was collected on Whatman GF/F glass fibre filters, washed with TCA and ethanol, and counted in a toluene-based liquid scintillation counting fluid. Radioactive materials were purchased from Daiichi Pure Chemical Co., Ltd, Tokyo, and The Radiochemical Centre, Amersham.

nucleosides into acid-insoluble material were measured after shift from 33 to 39 °C for mutant and wild-type cells. As shown in Fig. 2, a steady reduction with time was observed in the rates of incorporation of alanine and thymidine in *ts3* cells, the level reaching 30% after 12 h (Fig. 2*a* and *b*), whereas the incorporation of valine and uridine did not change much during a 12-h period in mutant cells (Fig. 2*c* and *d*). It has been shown with *ts3* cells that leucine incorporation is unaffected for the first 24 h at the elevated temperature¹. Wild-type cells, by contrast, incorporated an increasing amount of all these precursors into macromolecules as the incubation time was prolonged (Fig. 2). Although the decline of thymidine uptake was observed in parallel with that of alanine uptake in mutant cells, this is not necessarily an indication that the DNA replicating machinery is also thermolabile. Since simultaneous protein synthesis is a requisite for DNA replication to initiate and continue in eukaryotic cells³, we could argue that the decreased synthesis of DNA results from the reduced incorporation of alanine provided that alanine is essential to the synthesis of the proteins required for DNA replication. The result that the incorporation of alanine, but not of leucine or valine, was affected at the restrictive temperature indicates that an alanine-specific process is modified in the mutant.

In bacteria many mutants auxotrophic for an amino acid contain an altered aminoacyl-tRNA synthetase⁴. Mutants with a temperature-sensitive alanyl-tRNA synthetase incorporate alanine at a decreasing rate but continue to incorporate leucine

Table 1 Thermal inactivation of aminoacyl-tRNA synthetases of wild-type and mutant *ts3* cells

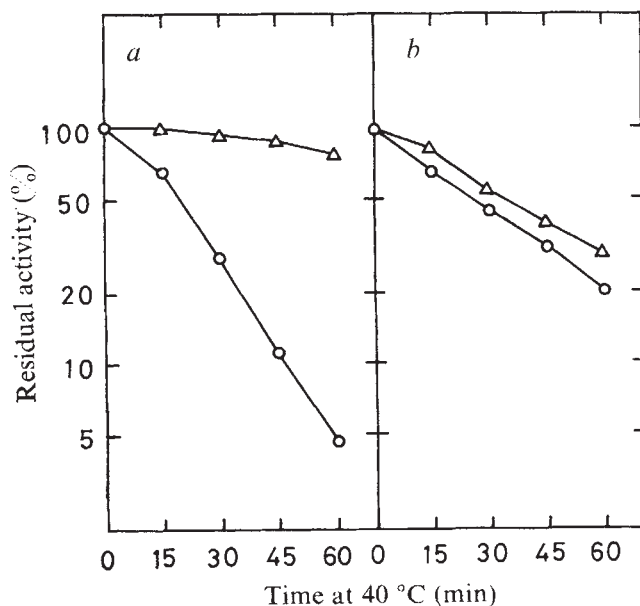
^{14}C -amino acid	Residual activity (%) after 60 min incubation at 40 °C	
	Wild type	<i>ts3</i>
Alanine	77.8	4.7
Arginine	12.2	11.7
Asparagine	65.4	67.1
Aspartic acid	53.2	79.1
Cysteine	38.5	28.6
Glutamic acid	12.4	13.7
Glutamine	16.0	12.8
Glycine	29.8	25.6
Histidine	15.3	14.4
Isoleucine	40.5	59.3
Leucine	28.9	19.9
Lysine	37.4	39.8
Methionine	75.3	87.3
Phenylalanine	59.9	60.6
Proline	8.4	8.1
Serine	62.3	54.7
Threonine	41.1	30.7
Tryptophan	78.7	94.5
Tyrosine	37.4	25.6
Valine	51.4	54.5

pH 5.2 fractions were prepared from wild-type and mutant cells and exposed to 40 °C for 0 and 60 min. Aminoacyl-tRNA synthetase activities were assayed for each amino acid as described in Fig. 3.

at a constant linear rate after exposure to the high temperature⁵. These findings suggest the possible involvement of alanine-activating enzyme in *ts3* cells.

The thermal inactivation of L-alanyl-tRNA synthetase (EC 6.1.1.7) was examined. The "pH-5.2 fraction" was prepared⁶ from mutant and wild-type cell extracts and exposed to 40 °C for various lengths of time. The residual activity was measured at 30 °C for alanine and leucine (Fig. 3). The alanyl-tRNA synthetase from *ts3* cells was far more rapidly inactivated than that from wild-type cells at 40 °C (Fig. 3*a*), whereas the leucyl-tRNA synthetases from mutant and wild-type cells were

Fig. 3 Thermal inactivation of alanyl- and leucyl-tRNA synthetases of wild-type (Δ) and *ts3* (○) cells. From cell homogenates, pH 5.2 fractions were prepared⁶ and used for assaying alanyl- (*a*) and leucyl-tRNA synthetase (*b*) activities after incubation at 40 °C for indicated periods of time. Each 0.1 ml reaction mixture contains: 100 mM Tris-HCl (pH 7.5), 10 mM KCl, 4 mM magnesium acetate, 5 mM ATP, 2 mM dithiothreitol, 10 μM each of 20 L-amino acids, 2 mg ml⁻¹ rat liver tRNA (Grand Island Biological Co., Grand Island, New York), 15 μM ^{14}C -alanine (159 Ci mol^{-1}) or 15 μM ^{14}C -leucine (280 Ci mol^{-1}), and cell extract. Reactions were carried out at 30 °C for 15 min and terminated by the addition of ice-cold 10% TCA. Precipitates were collected on Whatman GF/F glass fibre filters, washed and counted.



inactivated at approximately the same rate (Fig. 3b). The residual activities of alanyl-tRNA synthetases after 60 min incubation at 40 °C were 5 and 78% and those of leucyl-tRNA synthetases were 20 and 29% for mutant and wild-type cell extracts, respectively. These results demonstrate that alanyl-tRNA synthetase activity of *ts3* cells is far more thermolabile than that of wild-type cells.

To determine whether the altered function is alanine specific, the heat stability of the other aminoacyl-tRNA synthetases was studied. As shown in Table 1, there is little difference in the residual activities between wild-type and mutant cell extracts for all amino acids except alanine. The result clearly shows that the elevated temperature inactivates each aminoacyl-tRNA synthetase from wild-type and mutant cells to approximately the same level with the exception of alanyl-tRNA synthetase.

The extreme lability of the alanyl-tRNA synthetase of the mutant in *in vitro* conditions will explain the temperature-sensitive growth character of *ts3* cells. Further purification of the enzyme will reveal whether the affinity for L-alanine or the recognition site for tRNA is altered as shown in bacterial mutants^{7,8}. A Chinese hamster ovary cell mutant with a temperature-sensitive leucyl-tRNA synthetase has been reported⁹. There is a striking difference between the leucine mutant and *ts3* cells; valine incorporation is affected in the former but not in the latter in the non-permissive condition. The continued valine incorporation in *ts3* cells may represent synthesis of proteins that are low in, or lack, alanine or of abnormal polypeptide derivatives⁵. Uridine incorporation into RNA does not change much with time in spite of the depletion in alanyl- or leucyl-tRNA, which may be accounted for by assuming that either the pool of uncharged tRNA has not reached a level sufficiently high to cause a general shut-off in transcription or the stringent control does not operate in high eukaryotes. A decline in DNA synthesis is observed in both mutants and explained as a secondary consequence of reduced synthesis of initiator proteins of DNA replication. These points are, however, to be substantiated.

Non-conditional alanine-requiring mutants have been described¹⁰ which may carry a similar mutation.

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KOKI SATO

Department of Microbial Genetics,
Research Institute for Microbial Diseases,
Osaka University,
Yamada-kami, Suita 565, Japan

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three of these head proteins are found intact in the phage; the products of genes *E* and *D*, p*E* and p*D*, form the outer shell of the capsid⁶, and p*F*_{II} is thought to form part of the head–tail connector⁷. The other two gene products p*B* and p*C* undergo extensive alterations during head assembly. p*C* is fused to a minor fraction of p*E* and is cleaved to form two polypeptides, X1 and X2, which differ only in the length of the p*C* fragment present⁸. There are between three and nine molecules of each of these proteins in the phage head⁹. The product of gene *B*, p*B*, is also cleaved during head assembly to form p*B** (refs 4–6). There are between 10 and 14 molecules of p*B** in the phage^{3,6}. Since an icosahedron has 12 corners, it has been suggested that 12 p*B** molecules are structural components of the corners of the viral capsid^{9,11}.

We suggest, however, that the p*B** molecules are not distributed in the corners of the icosahedral viral shell but that, together with X1 and X2, they form the collar structure which connects the head and the tail. We have based these conclusions on the following observations. Petit λ particles (p λ), which appear in electron micrographs to be less angular in outline, and about three-quarters the diameter of λ heads (Fig. 1)¹², are normal precursors of mature λ heads^{11,13,24,25}. Particles similar in appearance, although not able to act as head precursors, can be obtained in equivalent amounts in the absence of p*B* and p*C* (refs 11, 14 and 15). According to the general principles regulating the construction of isometric viral shells, elaborated by Caspar and Klug, p λ particles must have 12 corners¹⁶. Therefore the presence of structures like p λ in *B*[–] and *C*[–] lysates indicates that p*B* and p*C* are not essential for the formation of corners. The less angular outline of all p λ relative to λ can be explained simply in terms of the grouping of the major structural proteins p*E* and p*D* on the surfaces of p λ and the mature head capsids as follows. In p λ , 420 molecules of p*E* are thought to be grouped as hexamers and pentamers to give 72 morphological capsomers^{17,24}. During maturation of the proheads, however, the p*E* molecules are regrouped as trimers and 420 molecules of p*D* are added to the shell, grouped as hexamers and pentamers, yielding a total of 212 capsomers one-half the size of the p*E*-containing p λ capsomers^{17,18,24}. The decrease in size and the increase in the total number of capsomers in λ heads could explain the observation that mature phage heads are more angular than p λ .

The collar structure observed in electron micrographs of λ heads (Fig. 1) is presumably derived from the head, since tails acquire collars only after their attachment to heads^{11,19–22}. Furthermore, it is likely that the collar is a preformed head structure and not simply a rearrangement of capsid proteins in response to the addition of a tail. Harrison and Bode (cited in ref. 21) have argued that such major structural alterations are unlikely in view of the low activation energy of head–tail joining reaction *in vitro* (8.5 kcalorie mol^{–1}).

A rough estimate of the size of the collar structure based on measurements from the electron micrograph in Fig. 1b gives a molecular weight of 1×10^6 . Thus, the five or six molecules of p*F*_{II} (molecular weight 12,000) present per phage⁷ could not make up the bulk of the material seen in electron micrographs (Fig. 1b). Furthermore a collar-like structure can be seen in phage heads and in p λ in *F*_{II}[–] mutant lysates (Fig. 1g). Clearly this structure must contain other head proteins in addition to the five to six molecules of p*F*_{II}.

The presence or absence of a collar-like structure in empty heads and in proheads can be correlated with the presence or absence of p*B**, X1 and X2 based on examination of the structures found in lysates of phage mutants. Empty heads found in lysates of a *Vam* tail defective mutant (Fig. 1c) have a structure which appears under the electron microscope to be very similar to the tail-collar

Model for arrangement of minor structural proteins in head of bacteriophage λ

THE morphogenesis and maturation of the head of bacteriophage λ requires the function of 10 phage genes^{1–5}, with the products of five of these genes, *E*, *D*, *F*_{II}, *B* and *C*, present as structural components of the head^{4,6,7}. The first

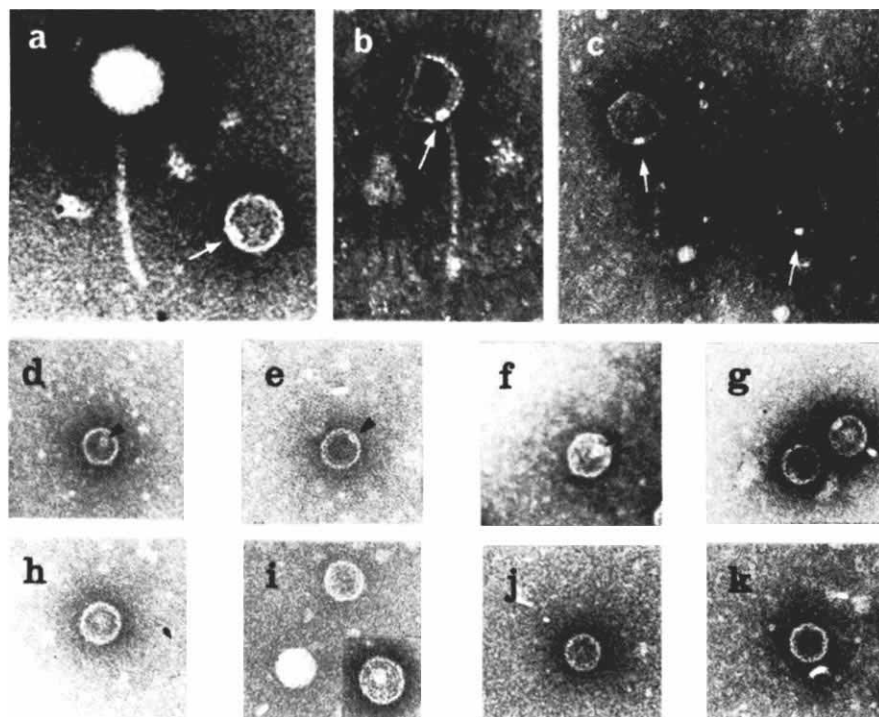


Fig. 1 Presence of tail collar in phage and p λ . Typical structures found in lysates with collar structures indicated by arrows. *a*, Lysate obtained after induction of 594(λ Cl₈₅₇Sam₇) showing a complete DNA-filled phage particle and a p λ with a collar. *b*, Ghosted phage showing collar joining tail to head. *c*, Empty heads from a lysate of 594(λ Cl₈₅₇Vam₇₅₀Sam₇). *d-g*, p λ with collar from lysates of induced cultures of: *d*, 594(λ Cl₈₅₇Aam₃₂Sam₇); *e*, 594(λ Cl₈₅₇Dam₁₅Sam₇); *f*, 594(λ Cl₈₅₇F_{1am}₇₈₅); *g*, 594(λ Cl₈₅₇F_{11am}₄₂₃Sam₇). A collar structure was clearly visible in about 30–50% of the p λ in the above lysates. *h-k*, p λ showing the absence of the collar, from lysates of induced cultures of: *h*, 594(λ Cl₈₅₇Bam₁₀Sam₇); *i*, 594(λ Cl₈₅₇Cam₄₂Sam₇); *j*, 594(λ Cl₈₅₇Bam₁Cam₄₂Sam₇); and *k*, 594(λ Cl₈₅₇Nu3am₈Sam₇). For a more complete description of the strains used, see refs 14 and 23. The preparation of the lysates and the procedures used for electron microscopy were described previously²³. *a-b*, $\times 160,000$; *c*, $\times 120,000$; *d-k*, $\times 100,000$.

found in disrupted phage heads (Fig. 1*b*). This structure is also found in p λ from wild-type lysates and from lysates of A^- , D^- , F_{11}^- and F_{11}^- phage-infected cells (Fig. 1*a* and *d-g*). Polyacrylamide gel electrophoretic analysis has indicated that all of these types of p λ particles contain pE, pB*, X1 and X2 (refs 9, 11 and 23). The collar-like structure is not found in p λ which do not contain pB*, X1 or X2, that is, p λ from lysates of B^- , C^- , B^-C^- or $Nu3^-$ phage-infected cells (Fig. 1*h-k*). Lysates of λC^- -infected cells contain three morphologically distinct p λ (Fig. 1*i*). Some appear completely empty, some appear completely full, and some have a small inclusion which is similar to the collar structure seen in wild-type proheads (Fig. 1*a*). In C^- p λ , however, this inclusion is larger and more diffuse than wild-type p λ collars, and may be unprocessed pB or pNu3 which has been found in these p λ (refs 9, 11 and 23). Since pE forms the outer shell of the p λ particle and is found in all types of p λ , regardless of whether they contain the collar-like

structure^{9,11,23}, it seems likely that the structure seen in Fig. 1*a-g* is made up of pB*, X1 and X2, rather than pE.

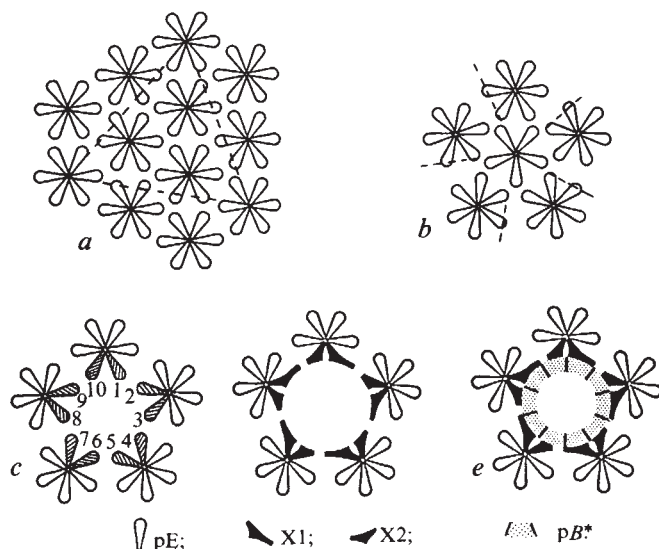
Previous data²³ suggest that pB and pC interact, and that the cleavage of pB requires the previous cleavage of pC. Thus it seems more likely that these proteins share a common function in the prohead, rather than being dispersed over the capsid.

These observations suggest that pB*, X1 and X2 form part of the head-tail connector. Figure 2 shows a model to explain how these molecules might be attached to a single corner of the head. The model proposes that the cleavage and fusion of pC and pE to form X1 and X2 takes place at only one of the 12 corners of the icosahedral shell.

Figure 2*a* shows a flat lattice structure of pE molecules grouped as hexamers as has been suggested for the molecular arrangement of the p λ capsid^{17,24}. Superimposed on this lattice is the outline of one face of the icosahedral structure. A corner can be generated from this planar hexagonal net with minimum distortion of the bond angles by cutting along one of the sides of the icosahedral face (Fig. 2*a*, broken lines) and folding the net into a cone to convert the corner capsomer to a pentamer¹⁶. The corner thus generated (Fig. 2*b*) consists of a pentamer of pE surrounded by five pE hexamers. The five faces of the icosahedron forming this corner are indicated by broken lines in Fig. 2*b*. If the pE pentamer is removed from this corner the resulting hole is surrounded by 10 molecules of pE (Fig. 2*c*, cross hatching). These 10 molecules of pE are not in identical molecular environments, but rather form two groups. One group, the even-numbered molecules in the figure, is oriented such that the left side of each molecule is facing the centre of the corner. The odd-numbered molecules have their right side facing the centre of the corner. We propose that 10 pC molecules are fused to these 10 pE molecules and are subsequently cleaved to form five molecules of X1 and five of X2 (Fig. 2*d*). The different molecular environments of the pE molecules could explain the presence of two sets of molecular species, X1 and X2, which have the same amino acid sequence but differ only slightly in length⁸. Ten molecules of pB* would then be located in contact with the ten X molecules to form the collar (Fig. 2*e*) seen in Fig. 1*d-g*.

Our previous results²³ suggest that pNu3 is involved in the assembly and functional cleavage of pB and pC. pNu3 has not been included in the model (Fig. 2) primarily because

Fig. 2 Model for the arrangement of the minor structural proteins of the head, pB*, X1 and X2, in the collar structure. See text for details.



it has a transient role in assembly and is not a structural component of the mature virion. It is possible that pNu3 provides a substructure to which pB and pC bind to form a precollar structure. This structure could then function as a prime corner which may serve as an initiation point directing the addition of pE to give a complete pλ. The processing of pB and pC would then produce the collar. This model would then provide a mechanism which would prevent pλ from developing more than one collar which might render them inactive in further head assembly. It might also provide an explanation for the formation of monsters in B⁻, C⁻ and Nu3⁻ lysates, which could be produced by the non-directed assembly of pE occurring in the absence of the pre-collar structure. B⁻, C⁻ and Nu3⁻ lysates contain large numbers of pλ; a prime corner is therefore not essential for pλ formation although it may be required for functional pλ assembly as the pλ isolated from these lysates do not act as preheads in the *in vitro* DNA packaging assay¹¹. There are insufficient data available to determine whether collars nucleate the assembly of pE or whether they are added to pre-existing pλ.

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HELIOS MURIALDO
PETER N. RAY

Department of Medical Genetics,
University of Toronto,
Toronto, Ontario, Canada M5S 1A8

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¹⁵N nuclear magnetic resonance of living cells

DIRECT observation of nuclear magnetic resonance (NMR) signals in living tissues, cells, or in their particulate components is an appealing aspect of NMR spectroscopy as it further extends its applicability from the chemical to the biological domain. Of the various nuclei available for NMR studies, ¹H (refs 1-3), ¹³C (refs 2 and 4) and ³¹P (ref. 5) have already been observed in complex biological systems. The potential of the technique for the study of dynamic metabolic processes, or, eventually, aspects of cellular physiological changes, has been demonstrated^{6,7}. No other spectroscopy affords the resolution and direct interpretability in terms of chemical functional groups that NMR provides. One drawback is its relative insensitivity, especially when the representative nuclides of biological interest occur in low natural abundance, for example, ¹³C, ¹⁵N. In the case of ¹³C, isotopic enrichment from suitably labelled precursors has been shown to provide most valuable

metabolic data in whole cell investigations⁸. There seem however, to be no studies of this type using nitrogen NMR. The relevance of nitrogen as a fundamental element of the constitutive building blocks of lipids, nucleic acids, proteins, and even some sugars, suggests that its direct observability in cells should provide an important probe to monitor the dynamics and control of its *in vivo* fixation and metabolism. We report here some results showing that ¹⁵N-NMR signals can be directly observed in whole, living cells.

The fungus *Ustilago sphaerogena* was grown at 30 °C in the low-iron media⁹ in which 95% ¹⁵N-ammonium acetate (Merck, Sharp and Dohme, Canada) was used as the sole nitrogen source⁹. Iron starvation, resulting in slow cell growth, was required to stimulate the production of ¹⁵N-enriched ferri-chrome peptides which are the subject of additional ¹⁵N-NMR investigations. The cells were collected and concentrated by centrifugation on day 17 after inoculation. Such cells are perfectly healthy as they can be routinely used as inoculum to start growth of new cultures. From 3.3 l culture medium the

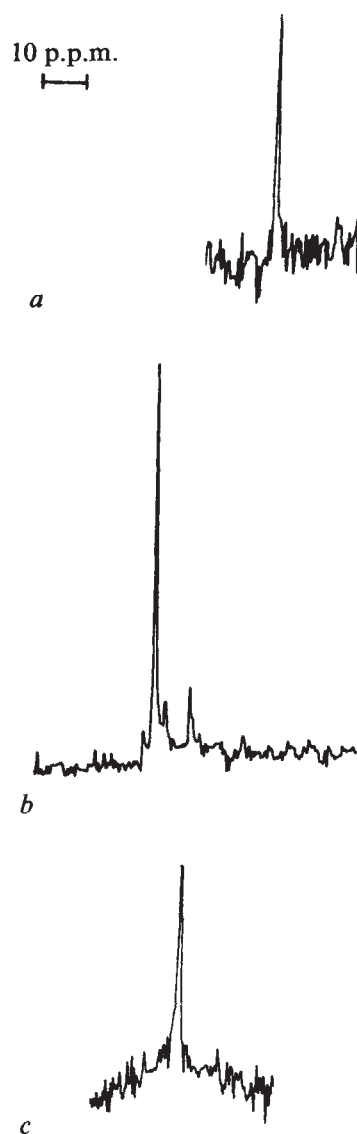


Fig. 1 ¹⁵N-noise decoupled Fourier transform ¹⁵N-NMR spectra at 10.1 MHz of an ¹⁵N-enriched culture of *U. sphaerogena*. a, Culture medium free of cells; b, freshly collected cell slurry; c, cell slurry aged for 9 d at 3 °C, and washed with distilled water before the NMR measurement. Spectra were taken at 32 °C on a Varian XL100-15 spectrometer. The chemical shift scale (p.p.m.) is referred to the external ¹⁵NO₃⁻ resonance measured in a separate tube containing a solution of 0.5 g ¹⁵NH₄¹⁵NO₃ in 3 ml D₂O/H₂O (Varian ¹⁵N standard 920 350-57). Each spectrum represents 120,000 pulse repetitions.

total yield of cells was a tightly packed pellet of about 10 ml. This material was divided into several batches, and both the cell pellets obtained by centrifugation and the growth medium were investigated using NMR.

In a first experiment about 2.8 ml of the packed cell slurry were deposited in a 12-mm NMR tube immediately after collection. A proton noise-decoupled ^{15}N -NMR spectrum of such a sample shows a group of four distinct resonances at -249.8 p.p.m., -253.8 p.p.m., -256.2 p.p.m. and -261.9 p.p.m., the amplitude of the signal at -253.8 p.p.m. being about ten times larger than that of the other resonances (Fig. 1b). No other resonances were observed in the spectral region from 30 to -470 p.p.m. To check for the possibility that some or all of the signals could arise from ^{15}N -labelled metabolites present in the growth medium external to the cell, a spectrum of the supernatant medium was recorded in the same conditions (Fig. 1a). A single signal was detected at -280.9 p.p.m., that is 19 p.p.m. more than the highest field signal arising from the cell pellet shown in Fig. 1b. This shows unequivocally that the signals in Fig. 1b arise from compounds within the cell.

In a second experiment, another batch of about 3 ml of the packed cell slurry was resuspended in the culture medium, and kept at 3°C for 9 d after collection. Thereafter the cells were separated by centrifugation, resuspended in distilled water, and left overnight at 10°C with continuous stirring. The cells were then centrifuged and transferred to an NMR tube as described above. The spectrum of such cold-aged, washed cells is shown in Fig. 1c. Only one signal was observed at -260.9 p.p.m., showing that at least three components present in the freshly collected cells (spectrum b) diffused out or were metabolised during the ageing period. Direct evidence for a metabolic inter-conversion comes from the observation that the strong line in spectrum c is in a spectral range which contained only b weak line in the freshly collected cells. Within the accuracy of the chemical shift determination it may well be that the signal in spectrum c and the line at -261.9 p.p.m. in spectrum b correspond to the same compound. Observation of resonances at 251.9 p.p.m., in the range of 145 – 155 p.p.m., and at 51.6 p.p.m. in the aqueous extract showed, on the other hand, that ^{15}N -metabolites leaked out during the wash.

At this stage the question arises as to the origin of the observed resonances. Overall, the frequency region in which the resonances are observed is typical for peptidyl amides. For example, the glycylglycine amide resonance is at -258.5 p.p.m. in the range $4 < \text{pH} < 10$, (ref. 10) and for a variety of other dipeptides and polypeptides the ^{15}N chemical shift typically varies from -270 to -230 p.p.m.¹¹. On the other hand, from chemical shift considerations the following representative compounds may be excluded: free amino acids (-290 to -350 p.p.m., excluding the ring nitrogens of histidine and tryptophan)¹¹, NH_4^+ (-354 p.p.m.)¹², $(\text{H}_2\text{N})_2\text{C}=\text{O}$ (~ -300 p.p.m.)¹³. The *Ustilago* ^{15}N signals are narrower and better defined than the resonances observed by Lapidot *et al.*¹⁰ in ^{15}N -labelled haemoglobin, which seems to be the only protein whose ^{15}N spectrum has been reported. Low molecular weight peptides exhibit relatively narrow ^{15}N resonances as exemplified by the natural abundance ^{15}N -NMR spectra of Viomycin and gramicidin S, a cyclopenta- and a cyclodecapeptide, respectively¹¹. The resonances shown in spectra b and c may thus quite likely arise from the low molecular weight peptide pool of the cell, intermediate in protein and peptide synthesis. In the growth conditions used here, *U. sphaerogena* is an active producer of the ferrichrome cyclohexapeptides, which contain a triornithyl sequence¹⁴. This suggests that the cells possess an active nitrogen metabolism in the region of the urea cycle so that it is not surprising that small ^{15}N -labelled metabolites should occur in sizeable concentrations.

The present study definitely establishes the suitability of ^{15}N -NMR as a non destructive tool for studying aspects of the cellular nitrogen metabolism, especially so since isotopic effects of ^{15}N , *vis-à-vis* the most abundant ^{14}N isotope, ought to be negligible. As with ^{13}C -NMR, improved instrumentation

and increased availability of labelled compounds should further enhance the appeal of ^{15}N -NMR for biochemical studies.

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M. LLINAS

K. WÜTHRICH

*Institute of Molecular Biology and Biophysics,
Swiss Federal Institute of Technology,
8048 Zürich*

W. SCHWOTZER

W. VON PHILIPSBORN

*Institute of Organic Chemistry,
University of Zürich,
8001 Zürich, Switzerland*

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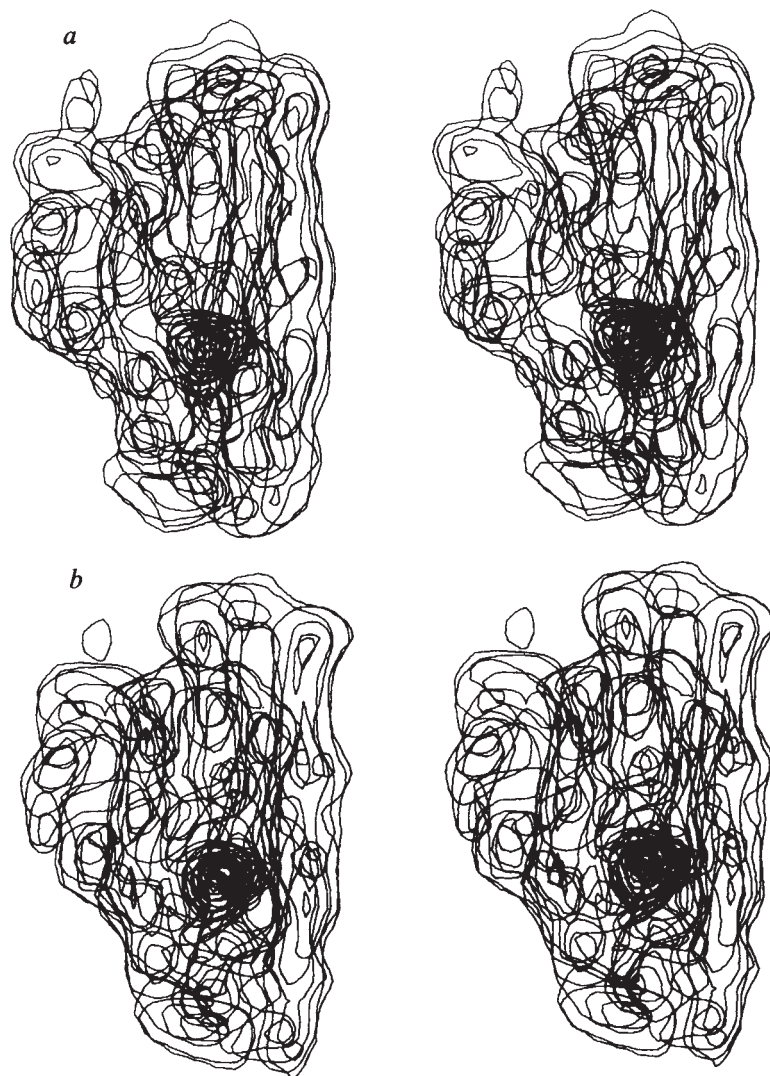
Quaternary and tertiary structure of haemerythrin

THE oligomeric protein haemerythrin is an oxygen-transport pigment found in erythrocytes of the coelomic fluid of certain invertebrates. It usually occurs as an octamer of molecular weight 108,000, in which each sub-unit contains two Fe atoms and reversibly binds one O_2 molecule¹. There is convincing evidence that myohaemerythrin, a monomeric protein found in the retractor muscles of the sipunculan worm *Themiste pyroides*, and the protomers of haemerythrin have quite similar tertiary structures^{2,3}. Consequently, the low resolution structure obtained for myohaemerythrin⁴ has been used to solve the structure of octameric haemerythrin by molecular search techniques.

Haemerythrin from the sipunculan *Phascolopsis* (syn. *Goldfingia*) *gouldii*⁵ is a hybrid molecule comprising five molecular variants, four of type A and one of type B, which differ only slightly in primary structure. Metazide-haemerythrin was dissociated and haemerythrin B monomers were isolated as described previously⁶. After purification by rechromatography, the monomers were reacted with excess cysteine ethyl ester to reconstitute octameric haemerythrin B (ref. 7). The protein was purified on Sephadex G-75 and concentrated by pressure ultrafiltration to give a 30 mg ml^{-1} solution. Tetragonal crystals were grown in sealed drops containing $5\text{ }\mu\text{l}$ of this solution and $2\text{ }\mu\text{l}$ of 2-methyl-2,4-pentanediol. The diffraction pattern from these crystals has Laue symmetry $4/\text{mmm}$ with reflections $00l$ extinct for odd l . The lattice constants measure $a=b=104.82\text{ }\text{\AA}$ and $c=54.08\text{ }\text{\AA}$. A more detailed description of the preparation of reconstituted octameric haemerythrin and of crystals grown from this material will be presented elsewhere.

The unit cell volume relative to the molecular volume of haemerythrin indicated that there must be two molecules

Fig. 1 Stereoplots of isolated electron density distributions (a) for a myohaemerythrin molecule and (b) for a haemerythrin protomer. The orientation for each corresponds to that of subunit 1 of Fig. 2. The myohaemerythrin map is a rotated version of one presented earlier⁴. The haemerythrin map is based on phases derived initially from the myohaemerythrin model and improved by a process involving the averaging of electron density related by non-crystallographic symmetry and the constraining of density in solvent regions to a constant level. The first contour level is at $0.53 \text{ e} \text{ \AA}^{-3}$ for myohaemerythrin and at $0.51 \text{ e} \text{ \AA}^{-3}$ for haemerythrin. Higher contours are drawn at intervals of $0.20 \text{ e} \text{ \AA}^{-3}$. Density values are on a quasi-absolute scale and include the F_{000} contribution. Although these density distributions for myohaemerythrin and haemerythrin are similar, significant differences are evident. The most pronounced structural difference appears at the CD corner and must reflect the five residue deletion in this region of haemerythrin compared with myohaemerythrin. The increased density near the beginning of helix C in haemerythrin may represent the bulkier side chains of residues (Glu, Phe, Tyr, Asp) which replace myohaemerythrin residues (Ser, Glu, Val, Val) at positions 68–71 (G. L. K., J. L., Cote, and S. E. Ludlam, unpublished). On the other hand, apparent differences at the start of the A helix may only be due to excess density from a close lattice contact in the myohaemerythrin crystal. It is interesting that the density appended at the first bend in the N-terminal arm, which decreased during the course of myohaemerythrin refinement, is much reduced in haemerythrin. These and other seemingly significant differences in the two maps indicate that the bias towards myohaemerythrin in the initial phase information for haemerythrin has been largely overcome.



in the unit cell to give a solvent content in the range typical of protein crystals⁸. Placements of two molecules in space group $P4_22$ are incompatible with the molecular symmetry and dimensions suggested by previous studies of haemerythrin crystals^{9,10}. Instead, the data are readily explained by two octamers centred about the special positions $(0, 0, 0)$ and $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$ in space group $P4_22$. Since the cell is not centred, the two molecules must differ in orientation by a 45° rotation about the c direction. Pseudo eightfold symmetry observed in $hk0$ precession photographs is consistent with this view. Exact $422 (D_4)$ symmetry of haemerythrin B is therefore revealed by this elegant arrangement which, in addition, uniquely defines the relative disposition of the two protomers in the asymmetric unit.

X-ray diffraction data from $\text{CuK}\alpha$ radiation were collected using a diffractometer to $5.5 \text{ \AA}$ resolution and corrected for absorption and the L_p factor. Initial phases for the observed structure amplitudes were calculated by Fourier inversion of a model structure composed from the electron density of isolated myohaemerythrin molecules appropriately oriented and positioned. An approximate orientation was determined by using the fast rotation function of Crowther¹¹ to correlate the observed intensity data with the squared transform of the electron density distribution in an isolated myohaemerythrin molecule. Attempts to ascertain the position using a translation function¹² were unsuccessful. Fortunately, a packing analysis limited the possible translational positions to three unique regions in the cell. Structure factors calculated from reoriented

myohaemerythrin molecules positioned at one of these possibilities satisfactorily matched the observed $20\text{-\AA}$ resolution data. This model was then improved by trial and error adjustment of the rotation and translation parameters in order to reduce the conventional R factor for data to $8\text{-\AA}$ resolution. Finally, the parameters were refined by a succession of fittings of the myohaemerythrin density to $5.5\text{-\AA}$ resolution Fourier maps of haemerythrin B based on phases from the current model. Details of the structure analysis will be presented elsewhere.

Several factors affirm the correctness of this structure solution. The final model structure gives an R value of 0.43 for all $1,141$ data. Packing contacts between subunits in this structure are stereochemically reasonable (Table 1) and consistent with biochemical evidence on possible subunit interfaces. Further credence is lent to the proposed structure by the strong density returned at expected iron sites in a Fourier synthesis of haemerythrin based on phases calculated from a myohaemerythrin model from which density for the dimeric iron centre had been removed. Also, the iron atoms of myohaemerythrin transform to positions close to multiples of $c/16$ which explains the great intensity of reflection $(0, 0, 16)$. Finally, Fig. 1 illustrates that the major differences in tertiary structures seen between these images of haemerythrin and myohaemerythrin correlate with the known differences in primary structure⁴.

The eight subunits of *P. gouldii* haemerythrin B are associated in 422 symmetry to form a molecule having approximate external dimensions of $75 \times 75 \times 50 \text{ \AA}$. The

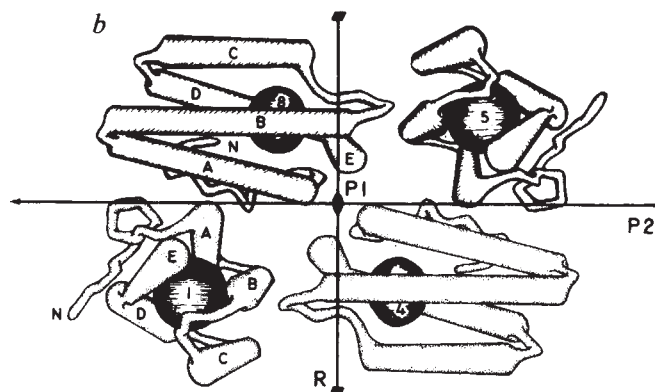
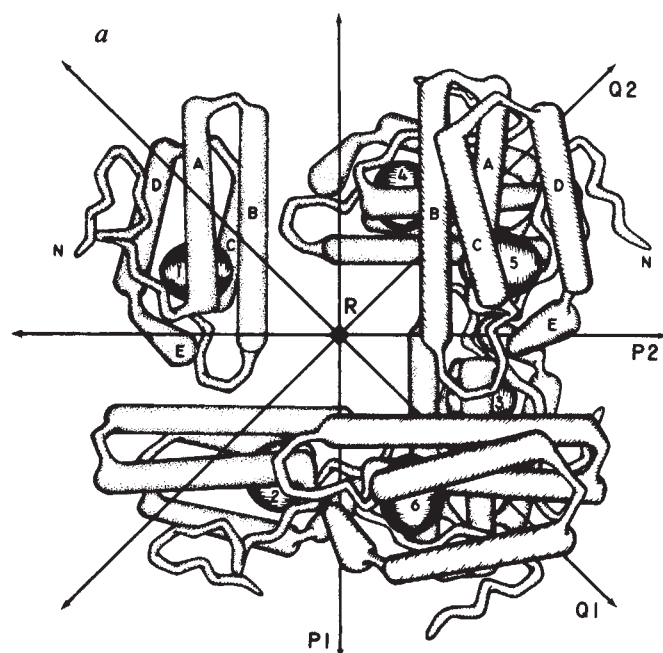


Fig. 2 Quaternary structure of haemerythrin B. Axes in a molecular coordinate frame are designated by P1, Q1, Q2 and R. For the molecule shown, diads P1 and P2 and the tetrad R are parallel to x , y and z , respectively, and intersect at $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$ of the crystallographic system. Subunits are drawn as schematic representations of the polypeptide chain. The amino-terminal arm is designated by N and helical segments are labelled A–E in accordance with the notation for myohaemerythrin⁴. The dimeric iron centres are represented by the oblate spheroids on which subunit numbers appear. Subunits 1–4 are numbered anticlockwise about R starting from protomer 1 in the third quadrant of the lower layer. Subunits 5–8 are related respectively to 1–4 by diad P1. Subunit 1 as viewed in *a* corresponds to the electron density distribution of the haemerythrin protomer shown in Fig. 1. View *a* is along R and shows the four protomers (stippled) in the lower layer and two of the four upper layer protomers (hatched) related by diads P and Q to the former. View *b* shows four subunits, two from each layer, as seen from within the octamer along the diad P1. Figures drawn from computer-generated schematics.

quaternary structure is illustrated in Fig. 2. Each subunit is oriented with its long (pseudo-diad) axis roughly perpendicular to the fourfold rotation axis (R). The assembly consists of two layers, each a square comprising four protomers related by R in an end-side arrangement. Layers are related to one another by the four twofold axes ($P1$, $Q1$, $P2$, $Q2$) which lie normal to R in a plane between layers. This formation produces a channel along R which swells to a chamber in the centre of the molecule. The chamber is 30 Å in diameter and 15 Å high and is bounded

Table 1 Subunit interfaces within and between octamers

Subunit pair and symmetry	Structural features with potential for contact	C_{α} pairs within 10 Å
R ($1 \rightarrow 2$)	BC (63–66) $\rightarrow$ B (45–46, 48–50, 52–53, 56–57) BC (64–65) $\rightarrow$ C (77, 81) E (107–108, 111–113) $\rightarrow$ B (42, 45–46, 49–50, 53)	42
R ($4 \leftarrow 1$)	Reciprocal to R ($1 \rightarrow 2$)	42
P2 ($1 \rightleftharpoons 7$)	N, A (17–19) $\rightleftharpoons$ N, A (17–19) N, A (17–20) $\rightleftharpoons$ E (111–113)	22
Q1 ($1 \rightleftharpoons 8$)	N (8–12) $\rightleftharpoons$ A (27, 30–31, 33–34, 38) N (11–12) $\rightleftharpoons$ B (43, 46) A (19–20, 23) $\rightleftharpoons$ B (46, 50) A (22–24, 26–27, 30–31) $\rightleftharpoons$ A (22–24, 26–27, 30–31)	71
$1 \rightleftharpoons 8'$ ($\frac{1}{2}, \frac{1}{2}, \frac{1}{2}$)	C (75, 78) $\rightleftharpoons$ C (75, 78)	3
$1 \rightarrow 6''$ (0, 0, 0)	A, AB, B (36–41) $\rightarrow$ N (1–3) CD (87–88) $\rightarrow$ N (1–2) CD (87–88) $\rightarrow$ D, E (103, 106)	15
$1 \rightarrow 7''$ (0, 0, 0)	N (1–3, 5) $\rightarrow$ A, AB, B (38–41)	7

All of the subunit interactions encountered by one protomer from the octamer centred at $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$ are tabulated here. These include four intramolecular contacts due to symmetry operations of the diads, P and Q, and the fourfold axis, R, and three intermolecular lattice contacts with subunits of the other independent octamer. The specific subunits and symmetry operators designated in this table refer to those identified in Fig. 2. Subunit 8' is related to 8 by a unit translation in z and subunits 6'' and 7'' are related to 6 and 7 by a 45° anticlockwise rotation about $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$ and translation by $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$. The contacts made by a protomer from an octamer at (0, 0, 0) are identical to those of one at $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$ except for lattice contacts reciprocal to those cited as $1 \rightarrow 6''$ and $1 \rightarrow 7''$ above. Structural features are designated by helix and corner letters⁴ in the notation of Fig. 2 with sequence positions⁸ of specific residues in parentheses. The identification of residues with potential for intersubunit contact was made by noting pairs of C_{α} atoms from different subunits which lie within 10 Å of each other. The C_{α} positions were derived from a tentative model¹⁴ for the polypeptide backbone of this protein. Those C_{α} atoms (25, 28, 29, 35, 43, 47, 51, 54, 55, and 62) which met the 10 Å criterion for potential contact but which were shielded by more superficial residues were excluded in this tabulation. The resulting number of non-shielded intersubunit C_{α} pairs within 10 Å of one another is given in the third column for each interface. The closest approach of an intersubunit C_{α} pair is 4.1 Å within a molecule and also 4.1 Å between molecules. The closest approaches between C_{α} atoms of protomer 1 and those of 3, 5, and 6 are 18.4, 26.4 and 25.1 Å, respectively.

by helices A and B and the E helical stub. The channel is lined by B and C helices and has a limiting aperture 20 Å square. Other access to the central chamber may be possible through eight small openings alongside the *P* axes (Fig. 2b).

Of the six possible types of subunit interactions in an oligomer with 422 symmetry¹³, three occur in haemerythrin. One type is the heterologous association between adjacent protomers related by *R* in a layer. Each subunit participates in two such interactions. The major isologous interaction is that between neighbouring protomers related by *Q* axes. Less extensive interaction exists between adjacent protomers related by *P* axes. The subunit interfaces in these associations are characterised in Table 1 by reference to a tentative model¹⁴ for the polypeptide backbone of haemerythrin. Several studies have implicated the involvement of certain residues at subunit interfaces^{7,15-19}. In agreement, this model has Cys-50 located near both the *Q* and *R* interfaces, and has Tyr-18, His-34, four lysines, and several carboxylates also placed at contact surfaces. In contrast, Tyr-70 and the variable residues^{6,20}, with the exception of Glu-63 and Val-9, do not occur near subunit contacts. The relative importance of the *P*, *Q* and *R* interfaces in stabilising the octamer is suggested by the contact ratio 22:71:84. Moreover, since the ratio of intramolecular contacts to lattice contacts is 177:25, the molecular structure is probably little affected by lattice interactions; thus averaging of crystallographically-independent subunits is justifiable at this stage of refinement. A more detailed description of the structure will be presented elsewhere.

Before completion of this investigation, we were informed that the structure of *Themiste dyscritum* haemerythrin had been solved by a single isomorphous replacement. While this manuscript was in preparation, we received a preprint²¹ describing those results. The two independent analyses have revealed essentially identical structures.

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KEITH B. WARD
WAYNE A. HENDRICKSON

Laboratory for the Structure of Matter,
Naval Research Laboratory,
Washington, DC 20375

GERALD L. KLIPPENSTEIN

Department of Biochemistry,
University of New Hampshire,
Durham, New Hampshire 03824

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Functional equivalence of the two iron-binding sites of human transferrin

FLETCHER and Huehns^{1,2} presented evidence that the two iron atoms bound to the serum iron-transport protein, transferrin are not equally available for the synthesis of haemoglobin by immature red blood cells (reticulocytes). They hypothesised that functional differences between the binding sites may serve to regulate the distribution of iron within the body³. Various studies (cited in ref. 4) have been published since, some of which support and some of which contradict this hypothesis. We devised procedures⁴ to compare the availability of iron at each site of transferrin for reticulocytes *in vitro*, as well as to compare the availability of iron in diferric and monoferric transferrin. In the homologous system of rabbit reticulocytes and rabbit transferrin, there was no functional difference between the two sites of diferric transferrin or between diferric and monoferric protein. In the heterologous system of rabbit reticulocytes and human transferrin, however, a difference between the sites of diferric transferrin and between diferric

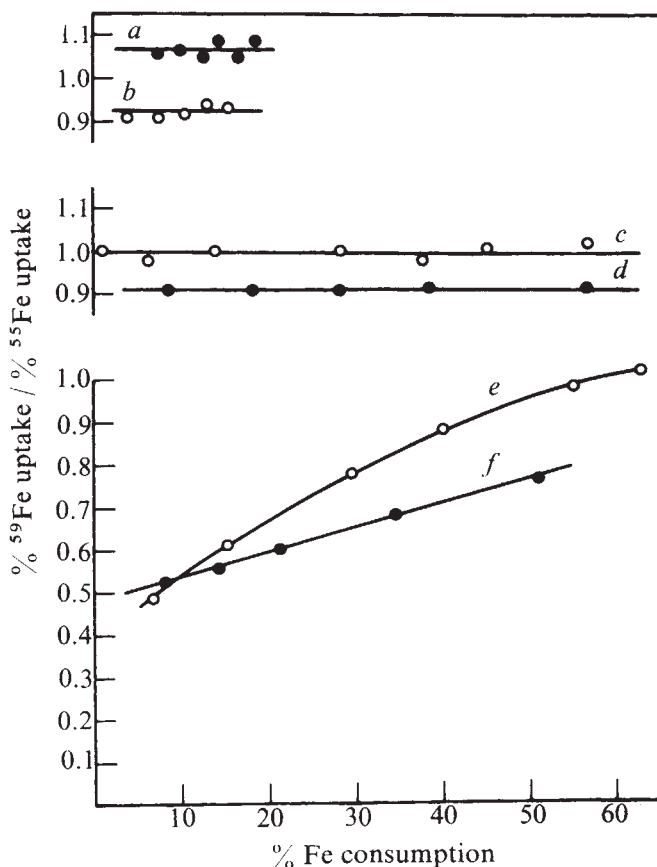


Fig. 1 Ratio of the uptake of ⁵⁹Fe and ⁵⁵Fe in double incubation experiments. *a* and *b*, Human cells plus human protein; *c* and *d*, rabbit cells plus rabbit protein; *e* and *f*, rabbit cells plus human protein. To increase the fraction of iron consumed, the concentration of transferrin in experiments with human reticulocytes was $\frac{1}{3}$ - $\frac{2}{3}$ that used previously in experiments with rabbit reticulocytes⁴. Haematocrits in incubation mixtures ranged from 25 to 50%. In the first incubation the ⁵⁹Fe consumed was: *a*, 20%; *b*, 59%; *c*, 22%; *d*, 39%; *e*, 25%; *f*, 46%. In experiment *a* blood from a patient with thalassaemia intermedia was used in the first incubation, and from a patient with haemoglobin C disease in the second. In experiment *b* each incubation made use of blood from different patients with sickle cell anaemia. The reticulocyte counts in the human blood ranged from 9 to 18%. Total incubation times in the second experiment were *a*, 120; *b*, 150; *c*, 35; *d*, 22; *e*, 60; and *f*, 32 min. In experiments *e*-*f* the incubation medium contained bovine serum albumin at a concentration of 2 mg ml⁻¹ before addition of cells. In experiments *a* and *b* albumin was omitted. Experiments *c* and *e* come from ref. 4.

and monoferric transferrin was observed. Those studies have now been extended to the homologous system of human reticulocytes and human transferrin. We found no difference in availability of iron at the two sites of human transferrin and only a slight difference in the availability of iron in monoferric and diferric protein.

To assess the relative availability of iron at the two binding sites, the following procedure was used⁴. Transferrin was treated with a quantity of ^{59}Fe -nitrilotriacetate sufficient to saturate 115% of the specific binding sites, and the excess iron was removed by gel filtration through Sephadex G-25 equilibrated with the saline incubation medium. After adding glucose to a concentration of 1 mg ml^{-1} , the ^{59}Fe -saturated protein solution was incubated with reticulocytes until 20–59% of the iron had been consumed by the cells. A quantity of ^{59}Fe -nitrilotriacetate calculated to be 10% in excess of that needed to replace consumed ^{59}Fe was then added to the supernatant incubation solution. After standing for 30 min at 20°C and 30 min and 37°C , the solution was passed through a column of Sephadex G-25 to separate unbound ^{59}Fe . The eluate was concentrated by ultrafiltration to approximately its original volume and glucose was added to a concentration of 1 mg ml^{-1} . This doubly labelled protein solution was then incubated with fresh cells and the ^{59}Fe and ^{55}Fe uptakes were measured. If site A were a better iron donor than site B, ^{59}Fe would be removed preferentially from site A in the first incubation and ^{55}Fe would preferentially occupy site A in the doubly labelled material. Therefore, a greater fraction of ^{55}Fe than of ^{59}Fe should be consumed in the second incubation. Alterna-

tively, if the sites function equivalently, the same fraction of each isotope would be consumed in the second incubation. To compare the availability of iron in monoferric and diferric transferrin, monoferric ^{59}Fe -transferrin obtained by isoelectric focusing was brought to saturation with either ^{59}Fe or ^{55}Fe and uptake of ^{59}Fe from the monoferric and both diferric species was compared.

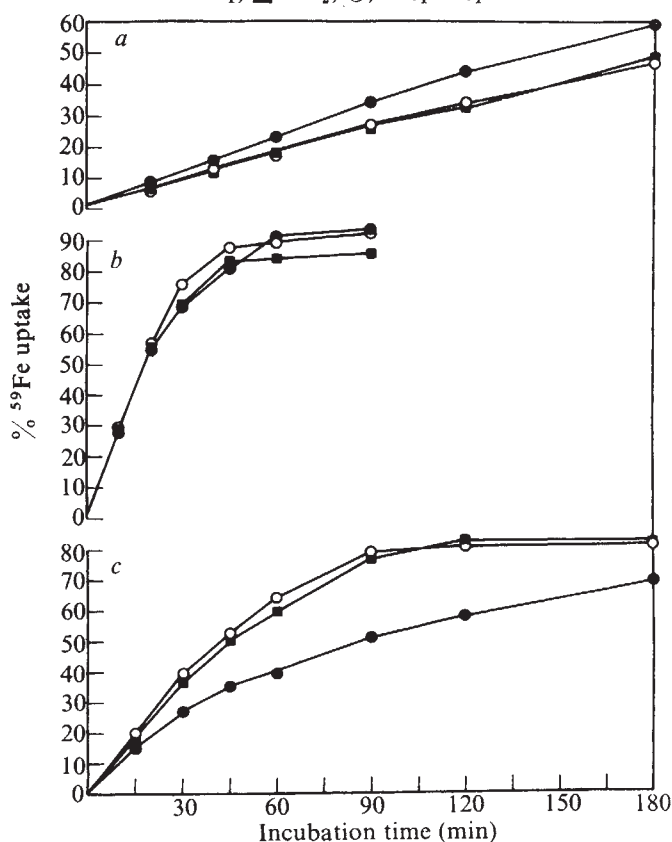
Results of the double incubation experiments are shown in Fig. 1. In both homologous systems (a–d) the ratio of the fractions of each isotope consumed is within experimental error of unity throughout each experiment, clearly indicating that one site of transferrin is not a better donor than the other. With rabbit reticulocytes and human transferrin (e and f) there is a preferential uptake of ^{55}Fe in the second incubation, as expected if one site is a better donor. Furthermore, the ratio of uptakes approaches unity with increasing total iron consumption since all of each isotope is potentially consumable. (Curves E and F are not expected to be identical because the fraction of iron consumed in the first incubation was not the same in each case.)

In Fig. 2 the availabilities of iron from monoferric and diferric transferrin are compared. Iron from monoferric human transferrin was slightly but consistently more available to human reticulocytes than iron from diferric transferrin in three different experiments, only one of which is shown. It should be noted, however, that since diferric transferrin carries twice as much iron as monoferric transferrin, it provides more total iron per unit time than monoferric transferrin. In the case of rabbit cells and rabbit protein, each iron atom of the monoferric and diferric species seemed to be equally available to reticulocytes. In the heterologous system of human protein and rabbit cells, monoferric transferrin was a somewhat poorer source of iron than diferric transferrin.

During our experiments we abandoned the use of albumin in the incubation medium when we became aware of its iron content. When analysed by atomic absorption spectroscopy, a solution of crystalline bovine serum albumin (10 mg ml^{-1} , Schwarz–Mann) contained $0.2\text{--}0.35 \mu\text{g Fe per ml}$. This would have been enough to affect the results of experiments comparing monoferric and diferric transferrin, were the iron available to the transferrin. Therefore, we studied the kinetics of iron uptake from monoferric human transferrin by rabbit reticulocytes in the presence or absence of albumin, or in the presence of albumin from which more than 90% of the iron had been removed by passage through a column of Chelex 100 (BioRad) equilibrated with 0.1 M sodium acetate, $\text{pH } 5.5$. No consistent effect of albumin on the rate of iron uptake was observed, so we cannot rule out the possibility that iron may have been transferred from albumin to transferrin in our conditions. Nevertheless, we think this unlikely in the absence of an iron-complexing agent to mediate transfer⁵.

Our results in Fig. 1 are inconsistent with the hypothesis of Fletcher and Huehns³. Experiments in support of this hypothesis are those of Awai *et al.*^{6,7} who studied iron uptake by rat tissue *in vivo*. The discrepancy between these results and ours may simply reflect the difference in species. We cannot however, account for the results of Awai *et al.* by a simple mathematical model. For example, consider the following experiment of theirs. Blood rich in reticulocytes was incubated with serum, in which the transferrin had been 90% saturated with ^{59}Fe , until only 35% of the iron remained. Assuming the greatest possible site preference, the remaining transferrin contained no ^{59}Fe at site A while site B still bound 70% of the ^{59}Fe originally present there. This was mixed with serum brought to 35% saturation with ^{55}Fe , so that sites A and B should each be 35% occupied by ^{55}Fe . In the resulting preparation, only ^{55}Fe should be present at site A, while a 2:1 ratio of ^{59}Fe to ^{55}Fe should be at site B. Even if a tissue has an absolute preference for site B, therefore, it should only consume a 2:1 ratio of ^{59}Fe : ^{55}Fe . Yet it was reported that this preparation delivered a 50:1 ratio of ^{59}Fe : ^{55}Fe to rat liver parenchymal cells (Table 1, in ref. 6).

Fig. 2 Comparison of iron-donating abilities of monoferric and diferric transferrin. Blood from a patient with sickle cell anaemia (11% reticulocytes) was used for the upper experiment, in which the incubation mixture contained 0.13 mg transferrin per ml and had a haematocrit of 38%. Qualitatively similar results were obtained with blood from patients with iron deficiency anaemia (9% reticulocytes) and pernicious anaemia (12% reticulocytes). All incubation solutions contained bovine serum albumin (2 mg ml^{-1}) before adding cells. The experiments with rabbit cells have been described previously⁴, and are shown here for comparison, a, Human cells plus human protein; b, rabbit cells plus rabbit protein; c, rabbit cells plus human protein. Transferrin: ●, $^{59}\text{Fe}_1$; ■, $^{59}\text{Fe}_2$; ○, $^{59}\text{Fe}_1$ $^{55}\text{Fe}_1$.



It may be significant that in most ferrokinetic studies in man, 95–98% of the plasma radioiron clearance can be accounted for by a single rate constant^{8,9}. If the tracer is bound randomly to the sites of transferrin, this would indicate that the sites are at least kinetically, and very likely functionally, equivalent in their iron-donating properties.

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DANIEL C. HARRIS
PHILIP AISEN

Departments of Biophysics and Medicine,
Albert Einstein College of Medicine,
Bronx, New York 10461

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Wavelength dependence of the bandwidths of visual pigment spectra

DARTNALL¹ proposed that the normalised absorption spectrum of any vitamin A₁-based visual pigment, when plotted on a frequency scale, could be fit to a standard shape, independent of its spectral absorbance maximum (λ_m). The existence of a standard shape implies that all pigments have the same band width. Dartnall devised an extremely useful nomogram so that given the λ_m of a pigment one could determine the entire shape of its main absorption band. A similar, slightly broader nomogram has been found for vitamin A₂-based pigments^{2,3}.

The validity of the idea that there are standard, λ_m -independent band shapes for all visual pigments, however, has been questioned^{4,5}. A clear trend has developed: spectra of blue-absorbing pigments tend to be broader than the nomogram, whereas red-absorbing pigments display spectra narrower than the appropriate nomograms. For example, the spectrum of the 432-nm frog cone pigment seems to be broader than the A₁ nomogram⁶. The action spectra of the 350-nm ultraviolet pigment and the 440-nm violet pigment in the moth *Deilephila elpenor* are clearly wider than the nomogram⁷; that of the 350-nm pigment is in turn considerably broader than that of the 440-nm pigment. Among the narrow, long wavelength spectra are those of the 620-nm tadpole pigment⁸, the 560-nm chicken cone pigment⁹, and the 625-nm goldfish cone pigment⁹. Smith and Pokorny⁵ have found that the nomogram does not describe the spectra of the long wavelength human cone pigments. In view of these findings, we investigated the theoretical guidelines governing visual pigment band shapes.

Several studies^{10,11} have described the spectral properties of the visual pigments in terms of those of two main classes of polyene molecules, cyanines and carotenoids. Cyanines, whose

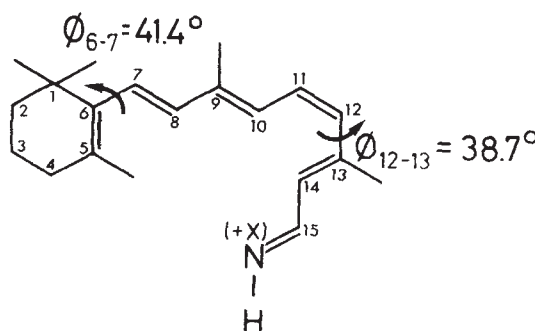


Fig. 1 11-*cis* retinal (protonated) Schiff base. X, Fraction of protonation $0 \leq X \leq 1$, as explained in the text. Values of the dihedral angles ϕ are from Gilardi *et al.*²³

π electrons are essentially completely delocalised, have approximately equal bond lengths and narrow bandwidths (about $1,000 \text{ cm}^{-1}$). In carotenoids, the π electrons are not completely delocalised; there is considerable single bond-double bond alternation and bandwidths are broad (about $6,000 \text{ cm}^{-1}$)¹². As a result of their bond alternation, carotenoids absorb at considerably shorter wavelengths than cyanines of comparable length. The amount of bond alternation also affects the width of the absorption bands¹³. If a given electronic transition causes a large displacement of the equilibrium nuclear configuration along a particular normal coordinate, a vibrational progression due to that normal mode will appear in the spectrum: the larger the displacement, the greater the extent of vibrational broadening. In polyenes, the first excited state is one of little bond alternation¹². Thus excitation of carotenoids involves a large displacement of the equilibrium nuclear configuration and a correspondingly broadened absorption band. In the cyanines, bond alternation is already indistinct in the ground state and so excitation results in only small displacements in the equilibrium nuclear configuration and little vibrational broadening.

In visual pigments, retinal is bound by way of a Schiff base linkage to the protein. The Schiff base, at least in vertebrates, seems to be protonated¹⁴ (for a review of the evidence, see ref. 4). There is a large red shift from the spectrum of free retinal to that of rhodopsin and other visual pigments. Calculations¹⁵ using the Pariser–Parr–Pople¹⁶ (PPP) technique show that this spectral shift can be associated with the formation of the protonated $\text{C}=\text{N}^+$ bond. The resulting chromophore has an increased degree of π electron delocalisation and a corresponding decrease in bond alternation. The process involves, in effect, conversion of the chromophore from a carotenoid (high degree of bond alternation, short λ_m) to a cyanine-like (little bond alternation, long λ_m) molecule. Differences in absorption maxima between pigments are attributed to secondary interactions with the protein^{15,17}. The expectation, based on this analogy with polyenes, is that a decrease in bandwidth should accompany any increase in λ_m (ref. 4). It would be of interest to determine whether these qualitative considerations are borne out by detailed theoretical calculations.

Table 1 Calculated and experimental wavelengths, calculated bandwidth parameters, and experimental bandwidths for several model compounds

Molecule	$(\text{CH}_3)_2\text{N}-(\text{CH}=\text{CH})_4-\text{CH}=\text{N}^+(\text{CH}_3)_2$	$\text{O}^--(\text{CH}=\text{CH})_4-\text{CH}=\text{O}$	11- <i>cis</i> retinal (at the crystal conformation of Fig. 1)	11- <i>cis</i> retinal Schiff base (at the crystal conformation of Fig. 1)
Reference	20	20	21	22
$\lambda_{\text{calc}}(\lambda_{\text{exp}})(\text{nm})$	623 (625)	540 (548)	339 (365)	347 (350)
$R(\text{\AA})$	0.0213	0.0358	0.0980	0.0957
$\Delta\nu_{\text{exp}}(\text{cm}^{-1})$	700	1,100	7,200	6,200

Calculations carried out in the standard PPP–MO technique¹⁶ in which carbon–carbon and carbon–protonated nitrogen overlap parameters were fit to match the spectra of the cyanine series $\text{N}^+=\text{C}_{2n+1}\text{N}$ (ref. 20). The carbon–unprotonated nitrogen overlap integral was chosen to reproduce the experimental spectrum of an 11-*trans* retinal Schiff base²². The carbon–oxygen overlap integral was chosen to reproduce the $\text{O}=\text{C}_{2n+1}\text{O}^-$ spectra²⁰. Details elsewhere (A. D. G., B. H., and T. G. E., unpublished).

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Table 2 Positions and bandwidth parameters for the λ_m in the protonated Schiff base, calculated at the crystal conformation of 11-*cis* retinal (Fig. 1)

Fraction protonation	$\lambda_m(\text{nm})$	$R(\lambda_m)(\text{\AA})$
0.0	347	0.0957
0.2	367	0.0901
0.4	416	0.0833
0.6	498	0.0746
0.8	600	0.0631
1.0	670	0.0485

McCoy and Ross¹⁸ have found a direct correlation between a theoretically-derived parameter R and the shapes of electronic bands in aromatic systems. The parameter R relates the displacement of the equilibrium value of the bond lengths to the intensity distribution within the vibrational envelope of a transition. With Δr_j equal to the excitational change in the j th bond length, they consider the quantity

$$R = \left(\sum \Delta r_j^2 \right)^{1/2} \quad (1)$$

Here Δr_j can be calculated from the ground and excited state bond orders using the standard¹⁹ relationship between bond length r_j and bond order P_j ,

$$r_j = r_j^0 - \xi_j P_j \quad (2)$$

where r_j^0 is the ideal single bond length and ξ_j is the empirically determined slope.

Parameters for the calculations carried out here were obtained by fitting theoretical transition energies to experimental absorption maxima for a number of polyenes related to the visual chromophore. Table 1 compares calculated and experimental wavelengths for some of these model molecules. The fit of the absorption maxima is quite good. Moreover, we note that the trend in the calculated values of R parallels the experimental trend in bandwidths; R is large for carotenoids and small for cyanines.

Calculations involving the visual pigment chromophore were carried out on the π electron system consisting of atoms 5–16 in Fig. 1. To investigate the bandwidth parameter over a large range in λ_m , we varied the fraction of protonation on the nitrogen, x , from zero to one (Fig. 1). Suzuki *et al.*¹⁵ have shown that the amount of charge on the terminal nitrogen can control π -electron density in the bonds and thus the amount of bond alternation. Varying the amount of protonation mimics the more complicated chromophore–protein interactions which cause the observed variations in λ_m . Results for the protonated Schiff base (Table 2) show that the experimentally observed trends are reproduced by our calculations: R decreases as λ_m increases. Moreover, the numerical values of R in the range of visual pigment absorption maxima are intermediate between those of cyanines and carotenoids but closer to the latter. This is consistent with the observation that the width of the nomogram is $4,300 \text{ cm}^{-1}$.

In conclusion, we have shown that the (protonated) Schiff base model of visual pigments predicts bandwidths which are wavelength dependent. Moreover, the observed trend in pigment bandwidths can be understood in terms of the qualitative considerations presented here. Thus, it is not surprising that it is impossible to fit the spectra of all pigments to a standard band shape. Rather, any nomogram should be applicable only to a limited range of absorption maxima; in the case of the Dartnall nomogram, this range is from about 470–530 nm.

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ALLAN D. GREENBERG
BARRY HONIG

Department of Physical Chemistry,
The Hebrew University,
Jerusalem, Israel

Department of Physiology and Biophysics,
524 Burrill Hall,
University of Illinois,
Urbana, Illinois 61801

Received June 19; accepted September 5, 1975.

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X-ray diffraction study of molecular structure and conformation of mezerein

THE crystal structure of one of the toxic components of the plant *Daphne mezereum*, mezerein, has been solved, unequivocally showing the molecular conformation in the solid state. Extracts from this plant have long been known to contain curative components, and have been used extensively in folk medicine in several countries¹. Mezerein has attracted attention for its anti-leukaemic activity against P-388 and P-1210 leukaemias in mice².

Many esters of phorbol (Fig. 1), which is closely related chemically to mezerein (Fig. 2), have also shown strong biological effects. Although these esters are reported to be extremely carcinogenic³, 12-hydroxy-daphnetoxin (Fig. 2) has no such activity. Furthermore, a range of esters of 12-hydroxy-daphnetoxin has significant antitumour activity⁴.

Because mezerein is the 5-phenyl-2,4-pentadionate ester of daphnetoxin, it would be interesting to know whether the attachment of this side chain changes the conformation of daphnetoxin or whether the biological activity is due solely to the added side chain. Also a knowledge of the architecture of this and related compounds will facilitate a systematic search for the biological systems affected by and the mode of action of chemical induction and inhibition of cancer.

Using a crystal of mezerein three-dimensional X-ray diffraction data were collected and reduced using conventional methods. The crystal belonged to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions $a=13.45 \text{ \AA}$,

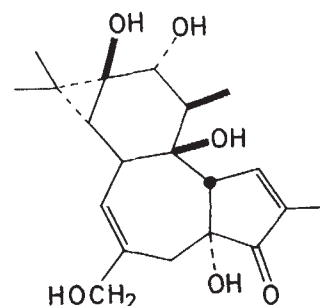


Fig. 1 Phorbol (the crystal structure of which has been solved by Brandl *et al.*⁵).

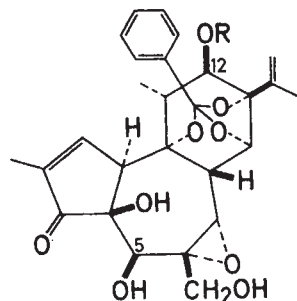


Fig. 2 12-Hydroxy-daphnetoxin (R = H), or mezerein (R = COCH=CHCH=CHC₆H₅).

$b=21.60 \text{ \AA}$ and $c=11.56 \text{ \AA}$, and contained one molecule per asymmetric unit. The structure was solved using direct methods and is at present refined to an R value of 0.131, using a full matrix least squares technique and isotropic temperature factors (details will be given elsewhere).

The molecular structure of the compound looks extremely compact, except for the 5-phenyl-2,4-pentadionate side chain. The fused-ring systems consisting of three, five, six and seven-membered rings form a cluster of roughly spherical shape (Fig. 3). The conformation of the central part of the molecule compares with that of 12-hydroxy-daphnetoxin^{5,6}. The cyclohexane ring is in a slightly twisted boat conformation. The structure of the cycloheptane ring can be regarded as that of a basal plane consisting of five carbon atoms of which the carbon-carbon bond involved in the epoxide ring is a member. At an angle of 110° to this plane the plane defined by the two bonds shared with the five-membered ring and the six-membered ring is attached.

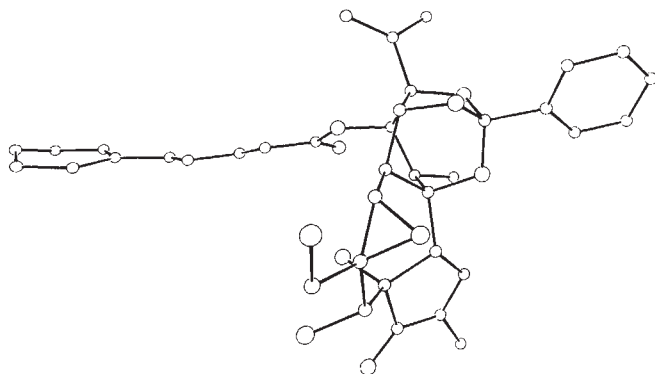


Fig. 3 A computer-generated drawing of the molecular structure of mezerein based on crystallographic data.

There seems to be conformational identity between the molecular structures of mezerein and that of 12-hydroxy-daphnetoxin tribromoacetate in the crystalline state⁶. In addition, due to the rigidity of the molecular framework caused by the attachment of various ring systems to the cycloheptane ring, it is most unlikely that the solution conformations are significantly changed.

We can thus conclude that the observed antitumour activity is somehow connected to the added ester at position 12. As Kupchan *et al.* pointed out⁴, the affixed ester may be involved in processes associated with cell wall penetration. In this connection it might be pointed out that the diterpene is in its fully extended form. An important question remains: at which step in the cellular metabolism is the toxic effect of mezerein and its analogue exerted, and which of the functional groups are active?

The crystal of mezerein used in this study was supplied by A. Groth and R. Söderquist, FOA, Stockholm, Sweden.

JENS NYBORG
TROELS LA COUR

Department of Chemistry,
University of Aarhus,
8000 Aarhus C, Denmark

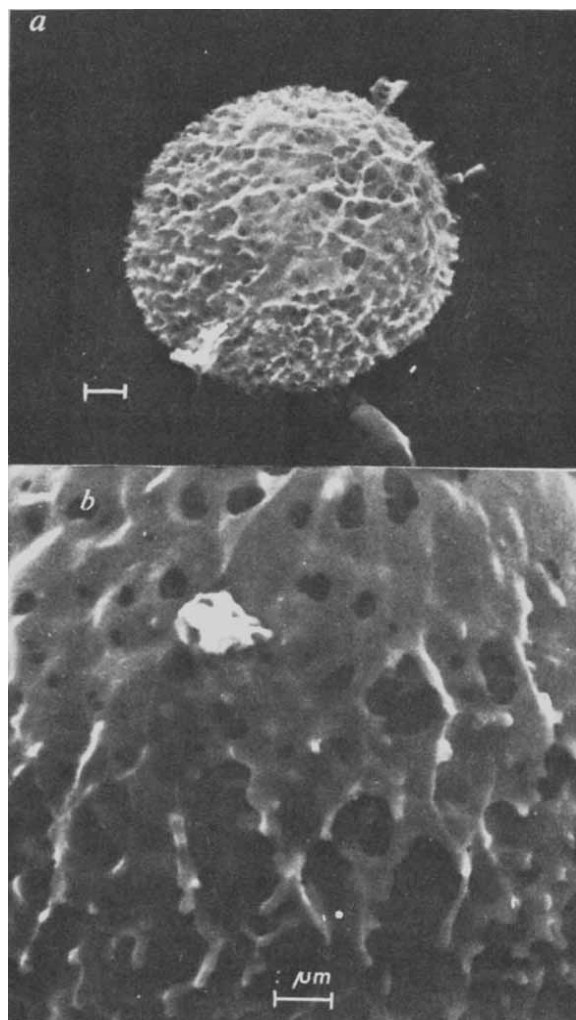
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Use of macromolecules in microparticles

POLYMERS of acrylamide, dextran, Agarose, and their derivatives have been used widely as matrices for covalent immobilisation of enzymes, for example, for biospecific affinity chromatography¹. Macromolecules can also be trapped in acrylic polymers, when added to the monomer solution before polymerisation²⁻⁴. Spherical polymeric particles are produced by homogenising the monomer solution with a suitable detergent in an organic solvent or mixture, in such a way as to produce a water-oil emulsion before polymerisation. The size of the polymeric particles will be directly proportional to the size of the droplets of the monomeric solution in the organic phase, giving a narrow size-distribution of the particles. The mean diameter can be varied greatly by changing the homogenising conditions. Hitherto, the aim has been to prepare particles with a mean diameter of 100-250 μm , to ensure a high flow rate when they are packed in columns. We have now found, however, that macromolecules trapped in the particles, retain their biological properties, and that therefore, microparticles

Fig. 1 Scanning electron micrographs. *a*, Polyacrylamide particle obtained by bead polymerisation; *b*, detail of microparticle. Bars, 1 μm .

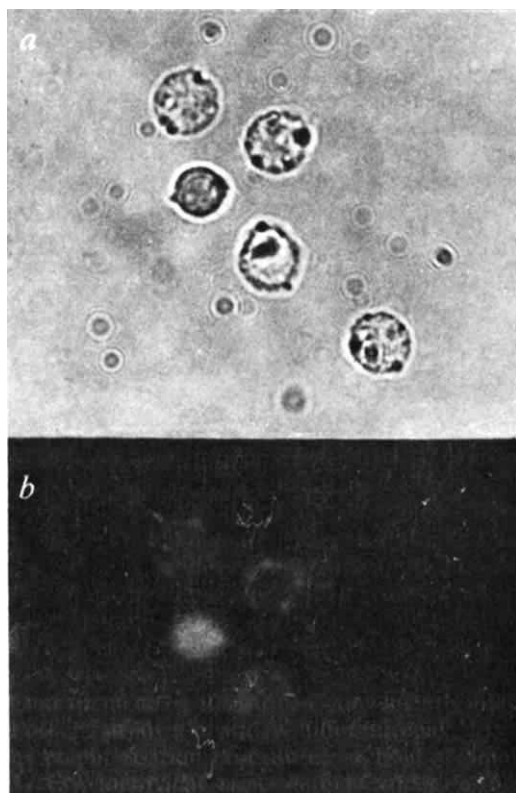


with mean diameter $<10\ \mu\text{m}$ can conveniently be used for several other purposes in widely different fields.

In the polymerisation procedure, we used acrylamide (1.5 g) and N,N' -methylene-bisacrylamide (0.5 g) in 25 ml of phosphate buffer, 0.005 M, pH 7.5, in which the protein ($10\text{--}100\ \text{mg ml}^{-1}$) was dissolved. The catalyst system, consisting of ammonium peroxodisulphate ($10\ \mu\text{g}$ in $50\ \mu\text{l}$) and N,N,N',N' -tetramethyl-ethylenediamine ($50\ \mu\text{l}$), was added to the monomer solution immediately before the homogenisation in toluene-chloroform (4 : 1, 300 ml). The detergent (Pluronic F68, Wyandotte Chemical Corp., Wyandotte, Michigan, 0.25 g) was dissolved in the organic phase. After the polymerisation, which was completed in about 10 min, the particles obtained were thoroughly washed with water and centrifuged. A more detailed description of the preparation procedure and the characteristics of the microparticles will be given elsewhere.

The amount of protein trapped, determined after acid hydrolysis, is directly proportional to its concentration in the monomer solution. Protein not trapped in the particles is found both in the water phase and the first washings. The polymeric network trapping the proteins is very porous, facilitating good penetration of the particles by reasonably large molecules, as is evident from Fig. 1. The equilibrium with the entrapped macromolecules is, therefore, reached very rapidly. Different macromolecule-ligand interactions, for example drug-protein or protein-protein interactions, can thus be easily studied using the microparticles. The material to be studied is suspended in an appropriate buffer, in which the particles with entrapped macromolecules will form a separate phase which can be isolated by centrifugation. During prolonged incubation in the buffer only insignificant numbers of macromolecules leak out of the particles. The particles can also serve as a matrix for different macromolecular reagents, for example in radioimmunoassays.

Fig. 2 Photomicrographs of fluorescein-labelled microparticles, containing concanavalin A, interacting with human erythrocytes, at pH 7.4 in 0.9% NaCl with 0.001 M CaCl_2 , 0.001 M MnCl_2 , 0.001 M MgCl_2 and 0.02% merthiolate. The photographs show the same cells in *a*, visible light; and *b*, blue light (about 490 nm).



The binding properties of the macromolecules trapped in acrylic polymers are unchanged. Thus, for instance, the binding of salicylic acid and warfarin to albumin is the same when immobilised in macroparticles as in equilibrium dialysis, with albumin in free form in the buffer. This enables binding studies to be performed in a suspension of particles, which can be isolated by centrifugation after brief incubation, obviating time consuming and laborious methods, such as equilibrium dialysis with semipermeable membranes. The use of microparticles containing albumin should greatly simplify the analysis of the drug-binding capacity of individual patient sera, enabling an individualised dosage regimen, using potent protein-bound drugs, to be achieved. Methods are being developed for this purpose, based on the competitive protein-binding principle.

A remarkable feature of the entrapped macromolecules is their availability on the surface of the microparticles, enabling them to interact with biological cells. Figure 2 shows how microparticles containing 2% concanavalin A (con A) and labelled with fluorescein isothiocyanate, have formed complexes with human erythrocytes. Particles not containing any con A will not show any significant affinity for the erythrocytes. In preliminary experiments it has also been shown that microparticles containing protein A from *Staphylococcus aureus*, known to react with the Fc part of IgG molecules⁵, are completely retained (at pH 7.4, in 0.1 M NaCl) on a Sepharose column, to which normal human immunoglobulin is covalently bound. Apparently, the polyacrylamide network allows sections of the protein to stick out of the microparticles, and to react with cells or Sepharose particles, but is compact enough to keep other sections of the protein molecules fixed in the particles.

The discovery that the macromolecules are accessible to specific membrane structures on the cell surface, opens up wide perspectives for the separation and characterisation of cells with specific surface structures. The separation of cells has hitherto been based mainly on centrifugation in density gradients, and results in bands of cells of the same density.

In a cell population of the same density, however, different cells can have different surface properties. Advantage has been taken of this to separate cells on, amongst others, nylon nets, to which biospecific molecules are bound⁶, or on biospecific Sepharose columns⁷. The microparticles, in which specific macromolecules are trapped, can be used for the same purposes. After a group of cells has been separated in a density gradient, the microparticles can be used to change the density of cells bearing the receptor for which the macromolecules have affinity. Repeated gradient centrifugation will then separate these cells. This general principle can be used in several different applications.

The microparticles can easily be labelled, with different fluorescent reagents, for example, large amounts of which can be bound to inactive macromolecules trapped in the particles. Combined with specific cell-markers (antibodies, agglutinins), such microparticles can be used to detect specific cell membrane structures using fluorescence microscopy. Such an application can be used in different cell studies, as well as for diagnostic purposes, the importance of which cannot yet be foreseen.

BO EKMÄN

INGVAR SJÖHOLM

Department of Pharmaceutical Biochemistry,
Biomedical Center, University of Uppsala,
Box 578, S-751 23 Uppsala, Sweden

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- ² Wada, A., and Kishizaki, A., *Biochim. biophys. Acta*, **166**, 29–39 (1968).
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matters arising

GFA protein in C-6 glioma *in vitro*

BISSELL *et al.* reported a 40-fold increase of glial fibrillary acidic (GFA) protein by radioimmunoassay in buffer extracts of C-6 glioma cells maintained in organ culture, compared with the same cells in monolayer and suspension culture¹. Quantitative data using buffer extracts should be interpreted with caution, since they may indicate differences in solubility rather than in total amounts. GFA protein is only partially soluble in water^{2,3}, and the factors regulating the ratio between water-soluble and insoluble fractions are still poorly understood. Solubility in buffer is greatly increased by *in situ* proteolysis⁴, and may be seen to vary considerably in different regions of the brain, even when a relatively insensitive assay such as double immunodiffusion is used.

¹ Bissell, M. G., Eng, L. F., Herman, M. M., Bensch, K. G., and Miles, L. E. M., *Nature*, 255, 633-634 (1975).

² Eng, L. F., Vanderhaeghen, J. J., Bignami, A., and Gerstl, B., *Brain Res.*, 28, 351-354 (1971).

³ Dahl, D., and Bignami, A., *Brain Res.*, 57, 343-360 (1973).

⁴ Dahl, D., and Bignami, A., *Biochim. biophys. Acta*, 386, 41-51 (1975).

⁵ Miles, L. E. M., Bieber, C. P., Eng, L. F., and Lipschitz, D. A., *Radio-immunoassay and Related Procedures in Medicine*, 1, 149-164 (International Atomic Energy Agency, Vienna, 1974).

BISSELL *ET AL.* REPLY—Bignami¹ raises the question whether our reported data on the quantitative increase of GFA protein in C-6 glioma cells *in vitro*² might not indicate differences in solubility rather than in total amounts. We welcome this opportunity to stress that the GFA protein estimations in our study were determined only on the phosphate buffer-soluble fraction. We have of course long been aware³ of the existence of water-soluble and insoluble GFA protein fractions, and we agree that the chemical relationship between the two and the

protein and of the stability and immunological identity of the purified and impure antigen, followed by a discussion on the soluble compared with insoluble GFA protein fractions, is to appear soon⁵. Contrary to Bignami's experience, our purified GFA protein standard (prepared from frozen human multiple sclerosis plaques) did not change in two-site IRMA immunoactivity for at least six months. In our report all samples were measured using the same standard.

Stanford University School of Medicine

¹ Bignami, A., *Nature*, 257, 827 (1975).

² Bissell, M. G., Eng, L. F., Herman, M. M., Bensch, K. G., and Miles, L. E. M., *Nature*, 255, 633-634 (1975).

³ Eng, L. F., Vanderhaeghen, J. J., Bignami, A., and Gerstl, B., *Brain Res.*, 28, 351-354 (1971).

⁴ Bissell, M. G., Rubinstein, L. J., Bignami, A., and Herman, M. M., *Brain Res.*, 82, 77-89 (1974).

⁵ Eng, L. S., Lee, Y.-L., and Miles, L. E. M., *Anal. Biochem.* (in the press).

Table 1 Solubility of GFA protein in different regions of the brain indicated by the immunodiffusion titre with GFA protein antiserum

	Water-soluble fraction	Urea-soluble fraction (first extract)
Cerebral cortex	1/4	1/4
Cerebral white matter	< 1*	1/32
Brain stem	1/8	1/16

Bovine brains were frozen at the slaughterhouse and GFA protein was extracted in 0.05 M sodium phosphate buffer, pH 8.0, with and without 6 M urea (w/v, 1:4).

* Precipitin lines were not observed against undiluted GFA protein antiserum unless the well was filled 2-3 times with the white matter extract.

As Table 1 shows, the white matter contains larger amounts than the cerebral cortex, which agrees with its relatively larger content of astroglia. However, the reverse is true for the water-soluble fraction, that is, only small amounts are extractable in buffer from the white matter compared with the cortex.

An additional point is that the radioimmunoassay for GFA protein⁵ is difficult to reproduce, since no information is given on the preparation of the standard. Our experience with micro-complement fixation indicates that GFA protein not only precipitates from the standard solution kept at 4 °C or frozen, but also that in these conditions the immunological activity per unit protein steadily decreases in the high-speed supernatant (100,000g for 1 h).

A. BIGNAMI

Department of Pathology,
Stanford University School of Medicine
and Veterans' Administration Hospital,
Palo Alto, California 94304

factors influencing their relative concentrations are still poorly understood. To assign, therefore, any special significance to either is premature. On the other hand, while we cannot exclude the hypothesis that variations in solubility could have played some role in determining minor deviations in the quantitative data obtained in our study, the overall reproducibility of the assays and the fact that they so unequivocally confirm previous observations on the same system using an immunofluorescence technique⁴ make it unlikely that solubility differences would account in any significant measure for the striking increases observed. The relevance of Bignami's table in relation to our report is questionable because the heterogeneity of astroglial cell populations that prevail in the different regions of the brain does not apply to the homogeneous cloned cell system used in our study.

A description of the properties of the two-site IRMA assay for the GFA

Tropomyosin and actin

BECAUSE of changes introduced before publication, Parry's letter¹ was not quite that to which my reply² was addressed. I wish to point out that his suggestion that tropomyosin would present pseudo-equivalent aspects to all seven actins is simply a restatement of my original hypothesis³. This hypothesis is strengthened by the observation of several charged groups in the sequence⁴ repeating at exactly 77 residues. The computed coordinates of these show that some could lie at the correct positions to interact with one strand of actin.

W. LONGLEY

Duke University Medical Center,
Durham, North Carolina 27710

¹ Parry, D. A. D., *Nature*, 256, 346 (1975).

² Longley, W., *Nature*, 256, 347 (1975).

³ Longley, W., *Nature*, 253, 126-127 (1975).

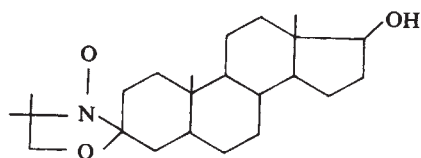
⁴ Stone, D., Sodek, J., Johnson, P., and Smillie, L. B. *Proc. 9th FEBS Meet., Budapest* (in the press).

PHA and lymphocyte membrane fluidity

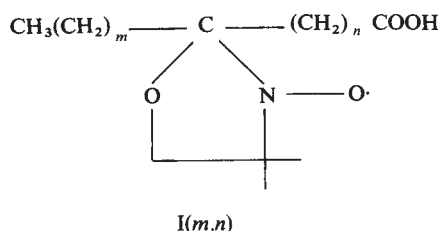
BARNETT *et al.*¹ reported that mitogenic lectins induce changes in the fluidity of lymphocyte membranes, which can be detected by means of the spin label electron spin resonance (ESR) technique. This finding, if substantiated, would be

of great importance in understanding the processes leading to mitosis. Considerable doubt has, however, been cast on the interpretation of Barnett's results, by the fact that the spin label used has since turned out to be a mixture of three compounds². Consequently, an independent study has been undertaken to examine the effects of the mitogen phytohaemagglutinin (PHA), using several different spin labels that probe different regions of the membrane.

The cells examined were human tonsil lymphocytes, separated (>90% purity) by teasing through a mesh and suspended in Hank's medium. ESR measurements were made using a Varian E-9, X-band spectrometer, equipped with a variable temperature accessory and a dual cavity operating in the H_{014} mode. Quantitation of the spectra was aided by the use of a Nicolet 1020A signal averager. Spin labels were purchased from the Syva Corporation, Palo Alto, California. These were the androstane label



and the stearic acid labels



where $m = 1$, $n = 14$; $m = 5$, $n = 10$ and $m = 12$, $n = 3$. All these labels are known to incorporate into the lipid regions of membranes. Two different methods were used to label the cells, namely deposition of the label on to the walls of a flask by evaporation of an alcoholic solution, and addition of $1 \mu\text{l}$ of a 10^{-1} M solution of label in ethanol to 1 ml of cell suspension. In both cases, cells were incubated with the label for 15 min at 37°C . They were then washed three times with medium, to remove free label and resuspended. A third method was also tried for label I ($m = 12$; $n = 3$), this being dissolved in an aqueous solution of sodium carbonate before addition to the cell suspension; however the method of introduction of label seemed to have no influence on the results obtained. Introduction of a spin label into the cells also had no detectable effect on their integrity. The PHA used

was Wellcome purified reagent. The optimum mitogenic dose for this material was found to be $1 \mu\text{g ml}^{-1}$ and this is the concentration used, except in one experiment where a higher dose ($10 \mu\text{g ml}^{-1}$) was used.

The androstane label and the stearic acid label I ($m=1$; $n=14$) both gave weakly immobilised, three-line spectra when added to the tonsil lymphocytes. The ratio of the heights of the three lines, which gives a measure of the mobility of the label³, was found to be unaffected by incubation with PHA for times up to 90 min. The label I ($m=5$; $n=10$) gave a complex spectrum showing both weakly and strongly immobilised components, possibly a result of some localisation of the polar nitroxide group in a hydrophilic region. Although difficult to analyse, this showed no detectable change with PHA. The label I ($m=12$; $n=3$) gave a strongly immobilised 'powder' spectrum when incorporated in the cells. From the separation of the extrema, an order parameter, S , was calculated⁴. This was determined with an accuracy of $\pm 1\%$ by means of the Nicolet 1020A. No change in the value of S could be detected, up to 90 min after the addition of PHA at 37°C . Use of a higher concentration of PHA also showed no effect on the value of S . When spectra were recorded at $21 \pm 1^\circ\text{C}$, the values of S were 0.678 ± 0.004 in the absence of PHA and 0.674 ± 0.007 in the presence of PHA. When measurements were made at 35°C , the fluidity of the membrane was increased and the value of S correspondingly reduced to 0.57 ± 0.01 .

In one experiment, cells were suspended in medium containing serum, in order to promote growth. As previously reported⁵, however, it was found that after introduction of spin label the observed spectrum was due primarily to label complexed with serum albumin.

Investigations into the fluidity of erythrocyte membranes in myotonic muscular dystrophy⁶ suggest that the spectral amplitude ratio A/B is more sensitive to change than the order parameter, S .

Matters arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

Consequently the present spectra were also analysed in terms of this parameter, but in no case could any change as a result of PHA be detected.

The present results indicate that PHA has no demonstrable effect on the fluidity of human tonsil lymphocyte membranes and it is unlikely that other human lymphocytes will behave differently. An alternative interpretation of the findings of Barnett *et al.*¹ must therefore be found. Since individual labels, in the mixture they used, would give differing values for the order parameter, the changes following mitogenic stimulation are consistent with a change in the relative proportions of the three labels present in the membranes. The cause of such a change could be as important as the apparent change in fluidity to which it was ascribed and this is now being investigated.

I thank Drs M. Moore and M. R. Potter of the Immunology Section for help and advice. The work was supported by the MRC.

N. J. F. DODD

Paterson Laboratories,
Christie Hospital and Holt Radium
Institute,
Manchester M20 9BX, UK

¹ Barnett, R. E., Scott, R. E., Furcht, L. T., and Kersey, J. H., *Nature*, **249**, 465-466 (1974).

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Chandler Wobble and viscosity in the Earth's core

AN addition is necessary to the references given by Dr Rochester¹. The (I_1, m_1) he is using are those of the reference² given as (1950a) on p.257 of *The Earth*, fourth edition.

BERTHA JEFFREYS

160, Huntingdon Road,
Cambridge CB3 0LB, UK

¹ Rochester, M. G., *Nature*, **255**, 655 (1975).

² Jeffreys, H., *Mon. Not. R. astr. Soc.*, **110**, 460-466 (1950); *Collected Papers*, 3, 547-553 (Gordon and Breach, London, 1974).

Erratum

In the original contribution by Rochester¹ the following text corrections should be made: para 1, line 12, $(\dot{I}_1^2 + \dot{m}_1^2)/\omega^2$. and para 2, line 17, $\gamma = (\dot{I}_1^2 + \dot{m}_1^2)^{1/2}/\omega$.

¹ Rochester, M. G., *Nature*, **255**, 655 (1975).

reviews

WAVES of various kinds are present in the atmosphere and the study of their generation, propagation and interaction is of great importance to meteorologists and aeronomers. Compressibility and buoyancy effects due to the action of gravity on vertical density gradients provide the main restoring forces for waves of comparatively short wavelength, with Coriolis effects increasing in importance with horizontal wavelength and becoming dominant for planetary-scale waves. Hydromagnetic effects due to the presence of the Earth's magnetic field complicate wave propagation in the electrically-conducting ionosphere.

Numerous observations of atmospheric waves are now available, thanks to the introduction of a variety of new techniques, including radar and acoustic echo sounders. The first of the three books listed above is an excellent systematic account of meso-scale and small-scale waves dominated by compressibility and buoyancy. It starts with an historical survey and particularly lucid discussions of the basic equations of fluid dynamics and of various theoretical concepts required in the study of wave propagation. Some boundary value problems arising in the analysis of wave-propagation in non-uniform media are then considered. Chapter 6 is largely concerned with various stability problems and the theory of the generation of atmospheric gravity waves, and chapter 7 with the theory of dissipative effects. Observations of gravity waves and infra-sound and

Air waves

R. Hide

Waves in the Atmosphere: Atmospheric Intra-Sound and Gravity Waves—Their Generation and Propagation. By Earl E. Gossard and William H. Hooke. Pp. xv+456. (Developments in Atmospheric Science, volume 2.) (Elsevier Scientific: Amsterdam, Oxford and New York, 1975.) \$64.95; Dfl 156.00. *Atmospheric Waves.* By Tom Beer. Pp. xvi+300. (Adam Hilger: London, October, 1974.) £16.00. *The Upper Atmosphere in Motion: A Selection of Papers, with Annotation.* (Geophysical Monograph No. 18.) By C. O. Hines and Colleagues. Pp. xii+1,072. (American Geophysical Union: Washington, DC, 1974.) n.p.

considerations of energy sources are presented in chapters 8–10, which include some particularly valuable case studies. Waves in the upper atmosphere are the subject of the eleventh and final chapter. This carefully prepared and well illustrated book can be highly recommended to graduate students and research workers in atmospheric physics.

The book written by Beer cannot, unfortunately, be highly recommended, owing to its many errors and muddled presentation of key ideas and results. This is a great pity because the publishers have gone to a great deal of trouble to produce

a handsome and well-illustrated volume, and there is certainly a need for a good treatise on the material covered, which includes treatments of waves on all scales.

Hines' book is altogether different from the other two, and it is by no means clear for whom the book is intended, apart from those specialists who may wish to possess in one volume a collection of excellent papers by one of the pioneers of research on waves in the upper atmosphere and his collaborators, together with a very personal and somewhat idiosyncratic commentary on each paper by the author. In the introduction to the book the author states that "... although I have by no means ignored my fellow professionals in making my commentary ... my thoughts were primarily with the student reader and my design was to provide him or her with a full view of the sort of workings that go on behind the development of a field. Scientific convention normally bars such exposure from orderly textbooks, but this unconventional format provided me with an unusual opportunity—one that normally arises in direct contact with students—and I have not hesitated to exploit it, hopefully for the benefit of students elsewhere". Some future historian of aeronomy will doubtless acknowledge the enterprise of the American Geophysical Union in making this material available, but few science students will have the time to pursue these fascinating byways. □

THERE are already available several comprehensive accounts of nuclear structure, but this volume is assured of a worthy place by reason of its clarity of exposition, attention to detail and coverage of the most recent advances.

The opening chapter is naturally devoted to the nucleon–nucleon force, and contains a very thorough account of its meson-theoretical basis. It is followed by a summary of our knowledge of the shapes of nuclei and of their electric and magnetic moments. Subsequent chapters are devoted to the various models that enable us to correlate and understand so many features of nuclear structure, starting with the statistical model and the associated

topics of the semi-empirical mass formula and infinite nuclear matter. There is a detailed account of the

Nuclear structure

P. E. Hodgson

Nuclear Structure. By William F. Hornyak. Pp. xii+605. (Academic: New York and London, July 1975.) \$49.50; £23.75.

single-particle model, showing how a simple one-body potential is able to account for so many features.

Substantial chapters follow on the individual particle or shell model and on the collective models. That on the shell model contains an

account of the coupling schemes and the general symmetry classifications, together with intermediate coupling and the cluster model. The account of collective models covers the classical liquid-drop model, the vibrational states of spherical nuclei and the collective states of deformed nuclei. Two concluding chapters are devoted to the electromagnetic interactions of nuclei and β decay, and there are appendices on angular momentum coupling and on the Wigner–Eckart theorem.

The level of presentation is intended to be that appropriate to a second-year graduate student, but the book will also be found valuable by senior undergraduates and established physicists. □

Proton-Transfer Reactions

Edited by E. F. CALDIN and V. GOLD, F.R.S.
1975: 456 pages: illustrated: 412 12700 8:
hardback: £18.00

Many of the fundamental concepts of chemistry – from the tunnel effect in chemical kinetics to catalysis by enzymes – owe their recognition or formulation to the study of proton-transfer reactions. The subject has been developed in extensive researches by many workers, and most notably by Professor R. P. Bell, whose book *The Proton in Chemistry* has become a modern classic of science. The present volume, dedicated to Professor Bell, aims to complement his book by providing a coordinated set of studies in depth on some of the central problems and growing-points of proton-transfer reactions, written by a group of leading workers in the field

Second Edition

Biochemistry of Antimicrobial Action

T. J. FRANKLIN and G. A. SNOW
Hardback: Second edition 1975: 240 pages:
7 tone and 78 line illustrations: 412 12900 0:
£7.00
Science Paperback: 412 12910 8: £3.95

This book provides a systematic guide to the field of antimicrobial biochemistry. The mechanisms by which antimicrobial agents have their effect are illustrated by a large number of examples, leading to a discussion of the development of drug resistance in micro-organisms. Extensive revisions have been made in this second edition and these include substantial changes in virtually every chapter. A new chapter has been added on the penetration of antimicrobial agents into cells.

Second Edition

Lipid Biochemistry

M. I. GURR and A. T. JAMES
Hardback: Second edition November 1975:
256 pages: illustrated: 412 13780 1: £6.50
Science Paperback: 412 13790 9: £3.95

This new edition has been thoroughly updated and the authors have added new material where appropriate – as, for example, the latest knowledge on the pathway for the biosynthesis of ether bonds. A new chapter *Lipids in food*, has also been added for the new edition. This chapter not only covers the biochemistry and physiology of dietary fats but also their role in the texture, flavour and aroma of foods. It also includes a discussion of diseases (heart disease, obesity, diabetes) in which dietary fats are thought to play a role.

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Circle No. 11 on Reader Enquiry Form.

Dynamic molecules

Molecular Reaction Dynamics. By R. D. Levine and R. B. Bernstein. Pp. vi+150. (Clarendon: Oxford; Oxford University Press: New York, November 1975.) £5.00.

THE last fifteen years have seen rapid progress in one of the most fundamental areas of chemical research—the awakening of our understanding of how reactions occur at the microscopic level. Indeed much of this work is so recent that an adequate grasp of the field can only be acquired after extensive perusal of the original literature and the relatively limited number of research reviews. It is for this reason that the appearance of Levine and Bernstein's *Molecular Reaction Dynamics* is particularly welcome at this time.

The book is directed at 'newcomers' to the field and contains material which has been presented orally to advanced undergraduates and, presumably, graduate students. The contents are organised into six chapters which make up a total of some 30 sections, most of which are subdivided further. The avowed aim is to provide a general feeling for the whole subject area rather than supply extensive detailed knowledge, and as such the volume is, as it sets out to be, much more of a 'course book' than a reference work. Chapter 1 serves to whet the appetite for the study of molecular dynamics, after which the logic is to initiate a detailed consideration of molecular collisions (chapter 2) as a prelude to an account of how scattering phenomena can be related to collision dynamics (chapter 3). This leads to a consideration of the trajectories across potential energy surfaces, the theoretical calculation of reaction rates, the relationship between micro- and macroscopic observations, the partitioning between the different forms of energy in the products (chapter 4) and a survey of the different forms of molecular energy transfer (chapter 5). The climax of this development is then experienced in chapter 6 in which the previous extensive preparation is applied to the experimental study and discussion of a series of chemical systems exhibiting respectively different types of interaction between the reacting species. The various sections of the book are adequately cross-referenced forwards and backwards.

The method of presentation is such that maximum value can only be derived from the text by reading it cover-to-cover a number of times, and a newcomer would only acquire a real understanding of some of the

mathematical material after a prolonged study of the relevant published literature. An annoying and distracting feature, which seems to be characteristic generally of 'beam' publications, is the extensive use of footnotes, often containing asides or amplifications of the discussion, which could readily be either omitted or incorporated into the main text. Apart from these objections, however, the book undoubtedly satisfies the need for a readable and comprehensive guide to this important area of study at an attractive price. **J. F. J. Todd**

Numbers

Transcendental Number Theory. By Alan Baker. Pp. x+147. (Cambridge University Press: London, April 1975.) £4.90; \$13.95.

A NUMBER is said to be algebraic if it is a root of a non-zero polynomial in one variable, the coefficients of which are integers, and to be transcendental otherwise. It is well-known that (in a measure theoretic sense) almost all numbers are transcendental, but to demonstrate that a specific number is transcendental is never trivial. The methods for solving this problem constitute transcendental number theory.

Professor Baker's tract is an account of the advances in this subject during the last decade. A short description of the origins of the theory is followed by an amalgam of the author's award-winning papers (Fields Medal) on linear forms in logarithms with some very interesting applications both in transcendence theory and in such other areas as Diophantine equations and Gauss's conjecture on discriminants of class number one. Further topics include Schmidt's theorem on simultaneous approximation of several algebraic numbers by rationals, Mahler's classification of transcendental numbers and Shidlovsky's theorem on algebraic independence.

The proofs of the major theorems are presented in a very economic style and can be fully understood only by students with a plentiful supply of pencil, paper and time. For such serious students the book will form an admirable introduction to this difficult field of research, being in addition a concise source book for the major additional developments and outstanding problems. Those wishing to obtain an appreciation of the scope of this field will also gain much from the more descriptive parts of the work. All readers of this timely and welcome tract will benefit from the appraisal of a pre-eminent contributor to the field. **D. A. Burgess**

Urban environmental management

Urban Environmental Management. By Brian J. L. Berry and Frank E. Horton. Pp. xv+425. (Prentice-Hall: Englewood Cliffs, 1974.) \$14.95.

BRIAN BERRY and Frank Horton have discovered a new way to produce large text books—the 'text with integrated readings'. They now follow their *Geographical Perspectives on Urban Systems* with a new text on urban environmental management. They produce a cross between a reader and a traditional textbook by using contributors' papers, as in the former, but integrating them with judicious introductory and linking texts of their own. This makes it possible to be the first in the field with a new book. But does it work?

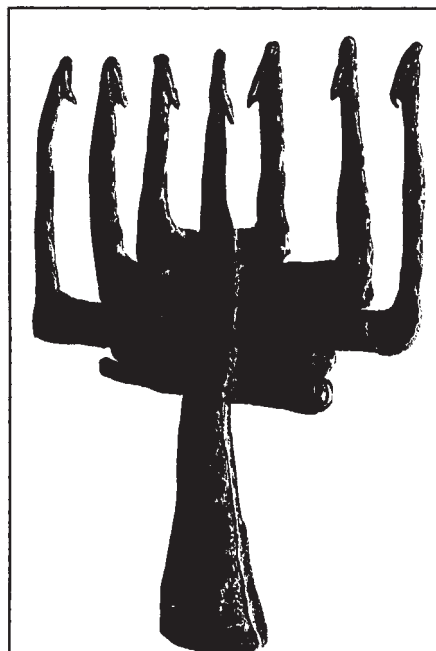
Although many of the contributing authors are first class, the present book is very uneven. Many of the articles used first appeared in that excellent journal *Science* and serve as thorough introductions. Most of the rest are either university discussion papers or government papers. These often contain masses of information, ranging in content, for example, from 'Generation of Solid Wastes from Five Major Sources in 1967' (covering the whole of the USA with no reference cited) to 'Licensed Swine Feeders in Northeastern Illinois' (reference given this time). Not surprisingly, it is very difficult for the reader to get all this into perspective. Absolute figures for 1967 do not say whether the problem is getting worse and if so at what rate.

Successive groups of chapters are concerned with environmental beliefs, environmental impacts of urbanisation, air, water, solid wastes, noise, the relationship between environmental pollution and health, and government programmes. Quite an agenda. The student is introduced to most of the basic elements of the various sectors, but he will sometimes, one suspects, have to be wary of the arguments put. For example, it is argued that particulate concentration increases with city size on the basis of a regression of the geometric mean of the pollutant against the log of the population, January temperature and the number of iron and steel production workers. Presumably, these were the best of the variables tried; the R^2 was 0.26. There are other examples of feeble analysis.

So the student gets his text a couple of years before he can have a 'proper' one, and lots of research workers who need a quick introduction to an important new field, can get it. But the price is fairly high. The quality of what is on offer varies tremendously, and the authors/editors have not always helped. They do indicate where a new reading starts, roughly, but not where it finishes, so it is not always

clear who is responsible for a piece of text—a bad principle to introduce. Many, if not most, of the entries in a long list of references are not actually cited in the text, and so the reader is not provided with an adequate guide to the literature. All in all, it may have been better for Berry and Horton to have been more patient before using their obvious skills in producing a conventional text.

A. G. Wilson



A fishspears from the Lipari Islands, off the coast of Sicily, probably used for tunny fish. Taken from *Beachcombing for Beginners* by Norman Hicklin. Pp. v+128. (David and Charles: London, 1975.) £3.50.

Insect muscle

Insect Muscle. Edited by P. N. R. Usherwood. Pp. xi+621. (Academic: London and New York, May 1975.) £14.50; \$38.25.

PHYSIOLOGISTS disagree as to whether diversity is a subject worthy of study in its own right but none would dissent from the view that a correct choice of experimental material is important for the investigation of basic biological phenomena. It has been amply demonstrated that insect muscle can provide many such opportunities. By contrast, it cannot be claimed that the study of insect muscles has yet made any important contributions to applied entomology. Workers who are interested in practical control measures can omit this book from their reading for the time being. It might become important introductory material for them in 10 years time when other aspects of insect physiology are better understood. Otherwise, however, this is a useful and timely book which should be read by all

general physiologists interested in the problem of muscular contractility.

Elder starts with a good review of all the significant, fine structural aspects of insects muscles. Finlayson deals with the difficult subject of development and degeneration, but in a book of this sort the articles should be more than a catalogue of original sources of information and should bring out points of significance. In fact, many of the articles meet this criterion.

On neuromuscular physiology, Osborne gives a good discussion of ultrastructural aspects and Usherwood and Cull-Candy provide a review of the difficult field of the identity of transmitters; their summary tables on the action of various chemical substances will be much used for reference. Piek, dealing with ionic and electrical properties, suggests that a highly active Na-K exchange pump operates in the extensive transverse tubular membrane, that this membrane is permeable to chloride and that this leads to a concentration of NaCl in the transverse tubular space which is high enough to produce a significant liquid junctional potential at its opening.

Aidley gives a short review of excitation-contraction coupling and mechanical properties, which is adequate in view of the fact that few significant differences have been discovered between insect and vertebrate muscles. Tregear presents a major review of the use made of a fibrillar muscle preparation for the elucidation of basic mechanisms of mechanochemical transduction; insect muscle had made important contributions to this general problem. Crabtree and Newsholme, on metabolism, return to an emphasis on diversity. Whereas the basic molecular mechanism for contraction seems to be the same for all muscles, differences in the type of fuel used for metabolic generation of ATP reflect the adaptation of the tissue to the needs of the whole animal, which are different for different insects. The article contains a lot of information and an interesting discussion of regulatory mechanisms and the nature and role of reversible mitochondrial shuttles. Hoyle, on neural control, is concerned with the programming of the output of motor neurones. Insect movement provides several well defined patterns of coordination, the analysis of which offers hope for a better understanding of the organisation of the central nervous system. This is an active and significant area of research. The last chapter, by Miller on visceral muscles, is a tasty *hors d'oeuvre* which, I hope, will stimulate the appetite of comparative physiologists for a field that cannot fail to integrate several aspects of insect biology.

The book is well produced and Professor Usherwood deserves our gratitude for undertaking the editorial tasks.

J. W. S. Pringle

Belts of ignorance

The Geology of Continental Margins. Edited by Creighton A. Burke, Charles L. Drake. Pp. xiii+1009. (Springer: Berlin and New York, 1974.) DM85.30; \$34.80.

FOR 600 million years the level of the North American continent as a block seems to have deviated by less than 60 m from the levels of the surrounding oceans. Yet during that time the Atlantic and Indian oceans came into being, the shape of the Pacific must have changed considerably, and the entire ocean floor has been renewed possibly twice or thrice. There must, therefore, be some mechanism which maintains a balance between the capacity of the ocean basins and the quantity of water available to fill them. It is very difficult at the moment to see what this mechanism may be. That is but one of the problems to which this book contributes.

The book provides a first rate summary of what is known about the continental margins—belts of ignorance as they have been termed—which extend sinuously across the Earth's surface for 350,000 km. These remarkable regions, which were necessarily ignored by the early landbound geologists and which have to some extent been left on one side by the recent rapid advances in our knowledge of the deep oceans, remain of critical importance.

It has long been known that sediments produced by the wearing away of continents contribute to the continental shelves, but what has become clear only in recent years is the effective way that such deposits are retained near the continents and the small extent to which they contribute to the sediments of the deep oceans. We now know of some of the ways in which sedimentary material is clawed back to form, once more, parts of a modified continental crust.

This book provides both a synoptic view of the margins of the present day continents and a conspectus of more general matters, such as bathymetry, the transition from continent to ocean, recent sedimentation, and the deformation of continental margins, all of which are treated in a score of contributions. The latter parts of this 1,000-page book deal with problems of the small ocean basins, such as the Black Sea or the Mediterranean, and with questions concerning ancient continental margins, the subject of a dozen or so papers. There is no index, but an excellent concluding article by the editors puts matters in perspective in an effective fashion, and the lay-out, consisting as it does partly of articles grouped under general headings, and partly of articles dealing with geographical regions, makes reference not too difficult. Nonetheless, in my opinion an index would have been a most useful addition.

One of the main virtues of this book arises as a result of the factual information that it contains, arranged as it is in a readily accessible fashion. But perhaps of greater importance are the discussions of still wider problems, which come up time and again. Why are continental margins situated where they are? How is it that, despite the great changes in the shape and distribution of oceans, the water rarely overflows on to the continents by more than a few tens of metres? One gets a vivid impression of the way in which geophysical evidence as to the structures of recent continental margins, supplemented by drilling, is bringing our knowledge to a point from which direct comparisons can be made with the remnants of ancient continental margins whose structures have been gradually pieced together by two centuries of geological enquiry.

From an economic point of view one is left with an intriguing question: whether petroleum may be present within the continental rise, the deepest lying part of the continental margin. Existing technology could probably be developed to recover hydrocarbons, should they be present, from the sediments of the continental rise, though these are covered by as much as 2–3 km of water.

All in all this is a first class book which does justice to the immensity of its subject.

J. Sutton

Aggression

Determinants and Origins of Aggressive Behavior. (New Babylon: Studies in the Social Sciences, vol. 22.) Edited by Jan de Wit and Willard W. Hartup. Pp. xiv+623. (Mouton: The Hague and Paris, 1974.) Dfl.84.

THE papers in this collection “were prepared for” or, alternatively, “grew out of” a NATO conference held in Monaco two years ago. The first and more modest estimate of their origins is that of the editors, whose thoughtful concluding paper testifies to the limits of growth that can be expected from such a conference. The 45 papers embrace a good range of current experimental approaches to the understanding of aggressive behaviour: the perspectives of ethology, physiological psychology, psychogenetics, social psychology, psychometry, pharmacology, psychopathology and anthropology are all represented to some degree.

In such a jamboree, coherence is almost inevitably missing. At least, the editors make little pretence of finding it: their own consideration of the effects of domestic punishment on children's aggression in other situations involves alarming discrepancy across just 11 pages; and at no point does their discussion owe anything to the one experimental report in the volume which explicitly concerns

Miller's displacement model of aggression. Perhaps out of deference to the many other assembled experts, few of the authors show much proselytising zeal for their own perspectives, and so the clarity born of dramatic confrontation is absent: even such giants as Berkowitz and Eibl-Eibesfeldt make few claims to each other's territory.

Nor are the editors helped by the largely unsurprising, if occasionally contradictory nature of the results which the papers report. Disagreements as to the effects of social isolation, of parental socialisation practices and of television violence rumble through these papers; but there are no dramatic breakthroughs. Perhaps the papers which best combine topicality and substance are those of Christiansen and Hutchings: by using both cohort twin-study and adoptive methodology, the partial heritability of criminality is reasserted; and Christiansen's data show that offences against the person are no exception.

Looking to the future, the editors finally opt to stress the need for “field studies”. But it seems an open question whether the armchair would not initially be a better location than the field if the laboratory does have to be left unmanned for a time. Both Hinde and the editors provide cogent illustrations of the diversity of aggressive behaviour and of the problematic nature of the concept of ‘aggression’ itself. Such a position doubtless reflects current academic wisdom in this area. But other papers—especially that by Olweus—note the way in which conceptually distinguishable ‘types’ of aggressive behaviour tend to appear together: across individuals, it can often be said that some are more generally ‘aggressive’ than others.

Trait and factorial approaches to aggression—especially if informed by the experimental and psychogenetic input which they have lacked in the past—would certainly help to settle some of the current confusion as to how many types or dimensions of aggressive behaviour are worth recognising. It is idle to rehearse for long ‘the multifactorial nature of aggression’ without using all appropriate methods of asking what the factors are and what proportion of natural variance they could possibly explain.

The general dimension of ‘aggressive behaviour disorder’ that seems so commonly in epidemiological work on individual differences remains largely unexplored in these papers; as do the processes of competition and conflict in human social hierarchies that might have had more relevance to NATO and more chance of incorporating the ethological notion of the normality of ‘aggression’. The volume is a competent and catholic sampling of the experimental studies of aggression conducted during the early 1970s; but it yields no very satisfying vision of the future.

C. R. Brand

Not quite the whole truth

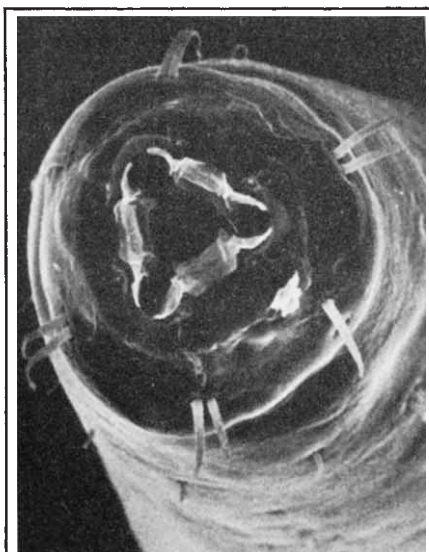
Neutron Stars, Black Holes and Binary X-ray Sources. (Astrophysics and Space Science Library, Vol. 48.) Edited by Herbert Gursky and Remo Ruffini. Pp. xii+441. (Reidel: Dordrecht and Boston, Massachusetts, 1975.) Dfl. 135; \$54.00 cloth; Dfl. 95; \$38.00 paper.

THE study of neutron stars and black holes, believed to be associated with many of the binary X-ray systems now discovered in space, has certainly progressed to the point where there is a need for a book pulling together the current state of the art in both observation and theory, and this volume fills the role, on the whole, admirably. The introduction by the editors provides a historical overview that is both comprehensive and a pleasure to read, but then the sense of anticipation with which the reader begins on the book proper is somewhat dashed by the terrible standard of English which he encounters: "Supernovae are the explosion of a star. This awesome and exciting realisation came about at the turn of the century from the following sequence of observations", and so on. In any multi-author book (this one is based on a session of the annual meeting of the AAAS held in February 1974), active editing is necessary to achieve a uniformly clear level of communication, and it is quite possible to achieve this without losing the character of the remarks of individual contributors. Am I alone in pleading for the abandonment of the Royal "we" in single author papers and in objecting to such circumlocutions as "while analysing the data on directions for that paper the author also came across information . . ." when he could say "I found"? Even one of the editors is guilty of jargon disease in an individual contribution, and as the price of books increases such irritations seem to become more noticeable and less excusable.

In terms of scientific content, however, this is a very good book. The emphasis on physical interpretation rather than on the erudite mathematics of general relativity is welcome, and although there is some repetition of ideas, especially from the different authors discussing aspects of the standard binary model, this helps in providing a better insight than if the model was discussed on a 'once-and-for-all' basis in a chapter of its own. One of the most remarkable features of the family of binary models is the patience with which a few dedicated theorists have worked out details for different mass ratios and configurations, even in the days before observations of binary X-ray sources gave an

impetus to work of that nature.

The chapter on observational properties of pulsars features the most complete table of pulsar parameters I have ever seen, covering 105 of these objects, and provides an early attempt to use this wealth of data for statistical investigations of pulsar properties. While the book was in preparation the discovery of a pulsar in a binary system opened a new phase in pulsar studies, and although E. J. Groth, the author of this section, might not agree it is in some ways convenient that his review should thus neatly tie up the pulsar package in the light of discoveries made before that identification.



Scanning electron micrograph of head of nematode worm, *Enoplus* sp.. Taken from *The Biology of Free-living Nematodes* by Warwick L. Nicholas. Pp. viii+219. (Clarendon: Oxford; Oxford University Press: London, August 1975.) £8.25.

The binary pulsar discovery paper is reproduced in an appendix, together with other contemporary and historic papers, a convenience for those readers too lazy to leaf through bound copies of journals in libraries. Other facilities for the lazy reader, however, are not so good. The index is inadequate, and the form of referencing is not uniform from one contribution to another. The editors seemed to have sacrificed something of the possible polish of the book for a breathless impression of urgent topicality, typified by the inclusion of the binary pulsar paper and an accompanying editorial comment. This is a trifle odd in view of the long publication time, and in my view quite unnecessary. The book cannot be expected to cover everything, and life would certainly be dull for the astrophysicist if the entire black hole story really could be wrapped up in one volume at this stage.

John Gribbin

Pathology of fishes

The Pathology of Fishes. (Proceedings of a Symposium.) Edited by William E. Ribelin and George Migaki. Pp. xii+1,004. (University of Wisconsin: Madison and London, September 1975.) \$35.00.

THE publication of this book represents a major step forward in fisheries biology. Until now, there has been little published in English on the diseases of fish. Indeed, the whole subject has been comparatively neglected; it is the decline in natural fish stocks under pressures of world fishing interests, combined with the rise of the fish-farming industry in the western world, which has given scientific and political recognition and impetus to this important field. It is encouraging to see how the increased importance of fish production has stimulated the members of many professions and branches of science to join the ranks of the traditional fisheries scientists.

This very substantial book is a record of a symposium sponsored by three of the pathology institutes in the US. It is essentially a medical textbook; the 39 chapters are arranged in six major parts to give detailed information on the specific diseases of fish, diseases of particular species, lesions of organ systems, chemical and physical agents of diseases, nutritional disease and neoplasias in fish. Each chapter has a substantial bibliography and the book is profusely illustrated with micrographs. There is a remarkable consistency of style and arrangement of material, and the editors and authors are to be congratulated on producing a coherent textbook from the various contributions.

There are still vast gaps in knowledge and this is exemplified by the very first chapter on comparative fish histology. In many ways this is the weakest contribution, and to a large extent reflects the lack of information on the structure of the normal fish. It deals in a superficial way with the information that is available, and this is unfortunate when so much of what follows is based on the interpretation of deviations from normal structure. To illustrate and supplement the text, the authors rely on the many plates and figures, the general quality of which is excellent; and some are outstanding, for example, those in the chapter on the infection of salmonid gills by amoebae. There is a particularly valuable part of the book dealing with chemical and physical agents of disease and a fascinating review of the extent and variety of neoplasias in fish.

The book should find a place not only on library shelves, but on the workbenches of fisheries biologists throughout the world.

F. G. T. Holliday

obituary

John W. Lyttelton, plant biochemist at the Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand died unexpectedly on May 30 at the age of 55.

A graduate from Auckland University (M.Sc., 1942) and Lister Institute, London (Ph.D., 1951) he was internationally known for his identification of fraction 1 leaf protein as ribulose carboxylase in the Calvin C4 photosynthetic pathway. Other achievements were his isolation of ribosomes from spinach chloroplasts and studies on plant saliva proteins as factors in cattle bloat. His contribution to New Zealand science ranged widely and included being organiser of Quantitative Biology meetings, Chairman of the New Zealand Biochemical Society, and Member of the National Committee of Biochemistry and Biophysics. He was elected a Fellow of the Royal Society of New Zealand in 1967.

Sir Eric Thompson, KBE, FBA, the world's leading authority on Ancient Mayan civilisations, has died in Cambridge at the age of 76.

After leaving Winchester College to serve in the army (under age) during World War I, he worked as a *gaucho* on a cattle ranch in South America. His interests in the civilisations there, particularly the Maya, led him to study under A. C. Haddon at Cambridge in 1924. He later went to work for Sylvanus T. Morley on the Carnegie Institute of Washington's major project of research and restoration at the great Mayan site of Chichén Itzá in Mexico. From 1926–35, he was on the staff of the Field Museum of Natural History in Chicago, during which time he carried out most of his purely archeological fieldwork. In 1927 he was seconded to the British Museum Expedition to British Honduras (now Belize), which was excavating at Lubaantum under the direction of T. A. Joyce. Fierce rivalry arose between the two men, but Thompson's conclusions were nevertheless published in the official report (together with comments from Joyce). At this time Thompson had discovered the important Mayan site of Busilha, near Lubaantum. He continued his researches by leading a number of other Field Museum expeditions to British Honduras, in the Cayo District and at San José, choosing a

smaller site in the hope that it might better reflect the nature of Mayan culture. From present-day Mayan descendants he was to cull an enormous amount of anthropological information about agricultural and religious practices and folklore, documenting the persistence of Pre-Columbian beliefs in a predominantly Catholic Society. By extrapolating backwards he hoped to illuminate the extremely fragmentary archeological record, and in fact threw much light on Mayan deities and the role of cacao and tobacco in ritual and commerce. Thompson joined the Carnegie Institution of Washington in 1935 and remained there until he retired in 1958. Under their auspices, he devoted much of his time to the decipherment of Mayan hieroglyphs, found on the numerous stone stelae at many sites, and comprising the most sophisticated means of recording and communication ever developed in the Ancient Americas.

One of his earliest achievements was to calculate the correlation, generally known as the Thompson Correlation, between the Mayan and Christian Calendars, which enables eleven events in the history of her civilisation to be precisely placed in time.

His monograph, *Maya Hieroglyphic Writing*, and his concordance, *A Catalogue of Maya Hieroglyphs*, will be essential reading for all future investigations. In 1972, he published a commentary on the earliest and most important of the three surviving Mayan manuscripts, the Dresden Codex. These were to comprise only a small part of a rich stream of deductive writing. In 1941 he was made Professor of the Museo Nacional de Mexico and in 1953 a consultant of the Centro de Investigaciones Antropológicas Mexicanas. On retiring to England he was elected a Fellow of the British Academy in 1959, and recently received a KBE. The Mexican government presented him with the Sahagun Medal in 1972.

Paul Lassenius Kramp, who died on July 13, in his 89th year, was the world's foremost authority on the cnidarian class Hydromedusae. His interest in marine biology was aroused while he was a student at Copenhagen University by three cruises on the 'Thor', and he used the wide practical experience that he had gained in sub-

sequent cruises.

His association with the University Museum of Zoology started in 1908, and when he retired in 1957 he was the chief curator of invertebrates. Apart from two early works (on chaetognaths, and fish eggs and young) practically all his work was devoted to hydroids and medusae. He published the classic work *The Hydromedusae of the Danish Waters* in 1927, where he pointed out the value of medusae as indicators of hydrographical conditions.

After a busy career, he published in his retirement three works which now form a necessary foundation for all engaged in this branch of science. These are *The Hydromedusae of the Atlantic Ocean and Adjacent Waters*, *Synopsis of the Medusae of the World*, and *The Hydromedusae of the Pacific and Indian Oceans*.

Professor **Erich Baer**, who died on September 23, aged 74, received his education in Germany. After his Ph.D. and further research in Berlin, he moved, first to Switzerland (in 1932) and then to Canada (in 1937). He stayed in the University of Toronto for the rest of his career, in the Chemistry Department initially, and then in the Banting and Best Department of Medical Research, where he was appointed professor in 1951. Among his many honours he gained the Chemical Institute of Canada medal in 1962, and the American Oil Chemists Society Award in 1964.

In his very active career, he published more than 150 papers in three main areas of research. His first success was the isolation of optically active 3-carbon compounds (particularly well-known is the 'Fischer-Baer' ester). Starting from these compounds he went on to synthesise phospholipids. For thirty years he worked in this field, developing elegant techniques for the synthesis of compounds of precisely known structure. Finally, he devoted the last ten years of his life to the synthesis of even more complex compounds—the phospholipids.

The availability of these synthetic compounds of precisely determined form was of immense assistance to biologists; the study of glucose and phospholipid metabolism were both greatly advanced by his work, as was the study of the role that phospholipids play in cell membranes and blood clotting.